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THE EFFECT OF BETA-CAROTENE ON THE ENZYMIC AND NON-ENZYMIC OXIDATION OF PEA LIPIDS

by

© GEORGE S. WALKER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF

MASTER OF SCIENCE

DEPARTMENT OF FOOD SCIENCE

EDMONTON, ALBERTA
SPRING, 1975

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled

"THE EFFECT OF BETA-CAROTENE ON THE ENZYMIC AND NON-ENZYMIC OXIDATION OF PEA LIPIDS"

submitted by GEORGE S. WALKER in partial fulfilment of the requirements for the degree of Master of Science.

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18 March 1975

Date

ABSTRACT

The influence of β-carotene on the oxidations of linoleic acid and lipids isolated from peas was studied in model systems. Conditions were selected to allow investigation of parameters influencing both enzymic and non-enzymic oxidations, with particular focus on the question of the role of this pigment as antioxidant or pro-oxidant.

The enzyme lipoxygenase was isolated from peas and characterized with respect to its elution patterns on Sephadex G 150 and DEAE-cellulose gels, activity as a function of enzyme concentration, susceptibility to calcium, cyanide, iron and copper; susceptibility to aging, substrate specificity, pH dependence, temperature coefficients, heat stability, molecular weight by disc gel electrophoresis, isoenzyme constitution, and its ability to destroy β-carotene in coupled oxidations with linoleic acid. Enzyme assays were performed by measuring with a Clark electrode the O_2 uptake by a linoleic acid substrate medium.

Matrices of pea albumins, pea globulins, cellulose, amylose, amylopectin and pectin were impregnated with neutral lipids (NL) and polar lipids (PL) from peas, with and without addition of β-carotene and freeze-dried. They were used as the basis for subsequent manometric studies of non-enzymic oxidations, comprising two-phase (solid-gas) systems. Measurements, made over 50 h periods at 50°C., were

carried out in the dark and in intense light.

Discussion of results of enzyme investigations marshalled arguments to support the conclusion that the storage resistant pea lipoxygenase which was isolated corresponds to isoenzyme-3 of soybean lipoxygenase, that it was not completely purified by the procedures employed, and that it is important as a so-called carotene oxidase, but not identical with it. Furthermore, carotene bleaching studies revealed that rate and amount of pigment destruction depend upon concentration of carotene and upon the ratio of its concentration to that of unsaturated lipid substrate.

Results of non-enzymic pea lipid oxidation investigations indicate that, regardless of matrix, β-carotene functions

1) antioxidatively in the light for both lipid categories (NL and PL) and in the dark for NL,
2) pro-oxidatively in the dark for PL.

Thus, although selection of a particular matrix influenced the amount of non-enzymic oxidation of pea PL or NL, β-carotene effected the oxidation, also, according to the lipid category and the presence or absence of light.

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I. INTRODUCTION

Among the constituents of foods susceptible to oxidation are the unsaturated fatty acids in their various forms of combination and numerous minor substances. The former usually include essential fatty acids and the latter, flavor and aroma constituents, pigments and vitamins. These oxidizable substrates can be accompanied by enzymes or other catalysts capable of accelerating their reaction with oxygen. Protective physical conditions and/or natural antioxidants may also occur.

In complex foods such as peas, oxidation of two or more substances may be coupled together, so that the oxidation product of one oxidizes another. The result is difficult to predict, but off-flavors and loss of nutritional value are certain. Organoleptic unacceptability is wholly or partly due to lipid deterioration.

Lipid breakdown can be hydrolytic but this is not usually important in terms of impairing food quality, except as a preliminary to subsequent oxidation. Serious lipid oxidation problems may arise in any food, especially - as in the pea - if a substantial proportion of total lipid is represented by unsaturated fatty acid moieties.

Disregarding the complexities of microbial spoilage, on the one hand, and residual metabolic (α-, β- and ω-) oxidation of the tissue, on the other, one can subdivide the oxidations of practical importance in food processing into three general categories:

1) Autoxidation
2) Oxidation resulting from the action of biocatalysts:
    a) hematin compounds
    b) lipoxygenases
3) Photoxidation

These are arbitrary, but justified by investigations into reaction mechanisms. Literature concerning photoxidations in foods or model systems is very scanty.

Ubiquitous atmospheric oxygen tends to attack the unsaturated fatty acid residues at the double bond, the more so if certain catalysts are present. "Peroxidation" (strictly, peroxygenation), the attachment of diatomic oxygen to a carbon skeleton, occurs initially. The possibilities of attack of oxygen at the substrate molecule are increased by numerous factors, chief among them being unsaturation. The number of secondary reaction products is almost boundless. Autoxidation of long-chain, unsaturated fatty acids and their derivatives is one of the most difficult and yet most investigated areas of organic chemistry technology.

The reason for stating that the above three categories were arbitrary becomes clear when the following points are considered:

1) Free radical mechanisms are generally recognized to play a central role in all three.
2) Autoxidation can be viewed as a branch of free radical chemistry, subject to catalysis by all known forms of electromagnetic radiation and corpuscular bombardment. However, photochemical peroxidation is non-autocatalytic whereas autoxidation is autocatalytic.
3) Certain pro- and antioxidants have a similar effect on all three types of reaction, although there is no universal agreement upon fundamental modes of interaction.

Since a food is a heterogeneous system containing both pro- and antioxidant substances, interpretation of reactions is complicated. Hence controlled conditions are needed to study oxidation parameters in isolation. The few reports available seem to indicate that β-carotene is a pro-oxidant in the light and an antioxidant in the dark, but work in our laboratory indicates discrepancies here.

The investigations reported in this thesis have been undertaken in an effort to discover trends in simple model systems which could reveal principles underlying oxidations occurring in the tissues of peas, and to contribute to characterization of the lipoxygenase isoenzymes present in our pea seeds.

II. REVIEW OF THE LITERATURE

A. Autoxidation.

The oxidative deterioration of food lipids involves primary reactions which are accompanied by various secondary reactions having oxidative or non-oxidative character. The principal mechanism involved in the primary oxidation of fatty materials has been fairly well elucidated, but a large number of secondary reactions are less well understood.

The reaction involves double bonds primarily, the hydroperoxide group appearing in α-position relative to a double bond. Saturated fatty acyl groups autoxidize to form hydroperoxides of undetermined structure, but the reaction is so slow as to be of minor importance.

1. Mechanism

The oxygen molecule is a diradical, $\cdot O - O\cdot$, with two unimpaired electrons. Autoxidation is a radical mechanism in which O_2 is used as the oxidant. Oxygen is, however, not reactive enough to attack the majority of organic molecules under normal conditions, but it combines easily with free radicals. The most common autoxidation is that in which compounds with labile hydrogens react to form hydroperoxides without prior cleavage of the O-O bond. This is energetically favored over cleavage of O_2.

The hydroperoxy radicals formed in the initiation step are thought to arise from a molecule-induced homolysis (Seakins and Hinshelwood, 1963):

$$R - H + O_2 \rightarrow R\cdot \ + \ \cdot O - OH \text{ (chain initiation)}.$$

and are more reactive than O_2 itself.

The chief concern here is the hydrogen of the general formula, R - H, which represents a fatty acid moiety. The probability of its being activated increases exponentially with increasing number of double bonds in the carbon chain. The process continues:

$$R\cdot \ + \ \cdot O - O\cdot \ \rightarrow \ R - O - O\cdot$$

$$R - O - O\cdot \ + \ H - R \ \rightarrow \ ROOH + R\cdot \rightarrow \text{etc.}$$

(chain propagation)

Breakdown of hydroperoxide can lead to different products according to conditions (such as pO_2):

$$ROOH \ \rightarrow \ ROO\cdot; \ RO\cdot; \ R\cdot; \ \cdot OOH\cdot; \ H\cdot,$$

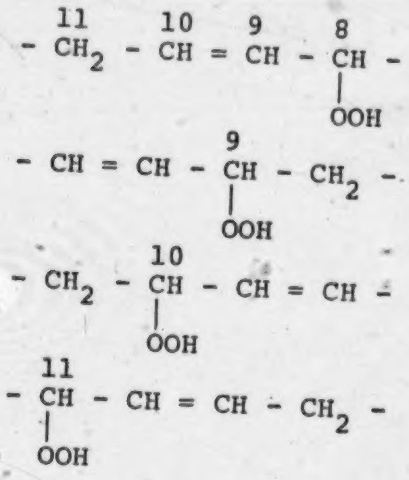
These are themselves capable of further catalysis. Thus autoxidation is autocatalytic (Rieche et al., 1963).

Chain termination occurs when two radicals recombine or by other means (see A.4, Antioxidants); e.g.,

$$\left.\begin{array}{ll} 2\ ROO\cdot & \rightarrow \\ ROO\cdot + R\cdot & \rightarrow \\ 2\ R\cdot & \rightarrow \\ 2\ RO\cdot & \rightarrow \\ \text{etc.} & \rightarrow \end{array}\right\} \text{(chain termination via non-radical products)}$$

A decade of intensive research was devoted to the investigation of kinetics, mechanisms and thermodynamics of the autoxidation of liquid olefins by E.H. Farmer, G. Gee, J.L. Bolland, L. Bateman and co-workers in the laboratories of the British Rubber Producer's Research Association.

According to the theory (Farmer, 1942; Farmer _et al_., 1942) autoxidation of methyl oleate should give rise to the following isomeric hydroperoxides:



$$-\overset{11}{CH_2}-\overset{10}{CH}=\overset{9}{CH}-\underset{\substack{|\\ OOH}}{\overset{8}{CH}}-$$

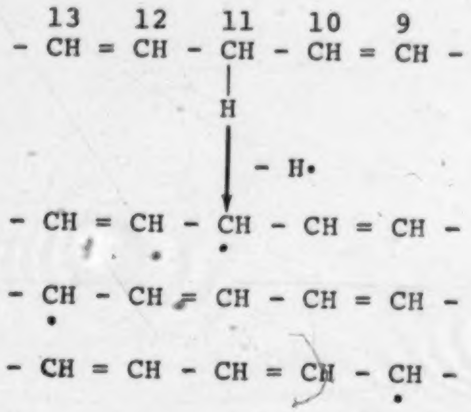
$$-CH=CH-\underset{\substack{|\\ OOH}}{\overset{9}{CH}}-CH_2-$$

$$-CH_2-\underset{\substack{|\\ OOH}}{\overset{10}{CH}}-CH=CH-$$

$$-\underset{\substack{|\\ OOH}}{\overset{11}{CH}}-CH=CH-CH_2-$$

Farmer studied hydroperoxide formation in autoxidizing ethyl linoleate, a particularly good model because hydroperoxide yield accounts for 98% of the oxygen uptake. The free radical chain reaction scheme, presented above, emerged from this and other work. The most striking feature is the inhibition of autoxidation by small concentrations of readily oxidizable substances which consume free radicals, but do not themselves undergo autoxidation via chain reaction.

The mechanism for autoxidation of linoleate based on Farmer's theory involves a homolytic abstraction of hydrogen from the pentadiene system, thus forming three possible resonance structures:



$$-\overset{13}{\mathrm{CH}}=\overset{12}{\mathrm{CH}}-\underset{\displaystyle\overset{|}{\mathrm{H}}}{\overset{11}{\mathrm{CH}}}-\overset{10}{\mathrm{CH}}=\overset{9}{\mathrm{CH}}-$$

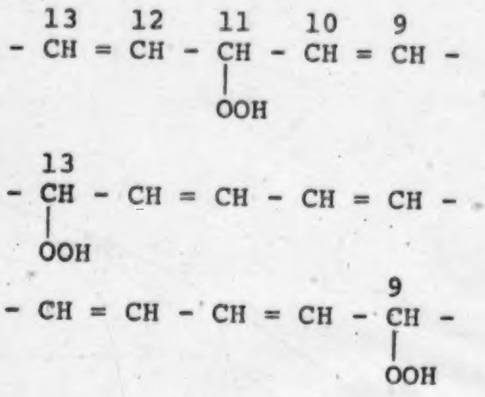
$$\Big\downarrow\ -\mathrm{H}\cdot$$

$$-\mathrm{CH}=\mathrm{CH}-\underset{\bullet}{\mathrm{CH}}-\mathrm{CH}=\mathrm{CH}-$$

$$-\underset{\bullet}{\mathrm{CH}}-\mathrm{CH}=\mathrm{CH}-\mathrm{CH}=\mathrm{CH}-$$

$$-\mathrm{CH}=\mathrm{CH}-\mathrm{CH}=\mathrm{CH}-\underset{\bullet}{\mathrm{CH}}-$$

which, upon propagation, become (theoretically)



$$-\overset{13}{\mathrm{CH}}=\overset{12}{\mathrm{CH}}-\underset{\displaystyle\overset{|}{\mathrm{OOH}}}{\overset{11}{\mathrm{CH}}}-\overset{10}{\mathrm{CH}}=\overset{9}{\mathrm{CH}}-$$

$$-\underset{\displaystyle\overset{|}{\mathrm{OOH}}}{\overset{13}{\mathrm{CH}}}-\mathrm{CH}=\mathrm{CH}-\mathrm{CH}=\mathrm{CH}-$$

$$-\mathrm{CH}=\mathrm{CH}-\mathrm{CH}=\mathrm{CH}-\underset{\displaystyle\overset{|}{\mathrm{OOH}}}{\overset{9}{\mathrm{CH}}}-$$

The formation of conjugated hydroperoxides consisting of approximately equal amounts of the 9- and 13-isomers from autoxidized linoleate indicates that reaction with O_2 at the end positions of the resonating system is favored.

This is expected since the products are thereby resonance stabilized by conjugated double bonds.

If a hybrid radical is postulated,

$$-\ \underset{\bullet}{\mathrm{CH}}.\mp.\mathrm{CH}.\mp.\underset{\bullet}{\mathrm{CH}}.\mp.\mathrm{CH}.\mp.\underset{\bullet}{\mathrm{CH}}\ -$$

cis,*trans*, *trans*,*trans*, or *cis*,*cis* configurations of the resulting hydroperoxides would become theoretically possible. Actually, *cis*,*trans* forms are in overwhelming predominance, since they are more stable. Current theories on fat autoxidation based on Farmer's free radical mechanism have been extended to explain the structures of the isomeric hydroperoxides obtained from oleate, linoleate and linolenate by means of this sort of hybrid radical. Assuming oxygen to attack at either end of the resonating system is enough to account for all the hydroperoxide structures identified in the literature (Cf. Pezold, 1969).

Further research by this group into autoxidations of linoleate at several different temperatures indicated that the rate of oxidation was directly proportional to linoleate concentration. Bateman *et al*. (1953) found that above a certain level of oxidation, the rate of oxidation increased in a straight line relationship with the extent of oxidation (Figure 1). That hydroperoxides themselves were catalysts, was inferred from the fact that all of the absorbed oxygen appears, initially, as hydroperoxide. Seakins and Hinshelwood (1963) provided evidence for the

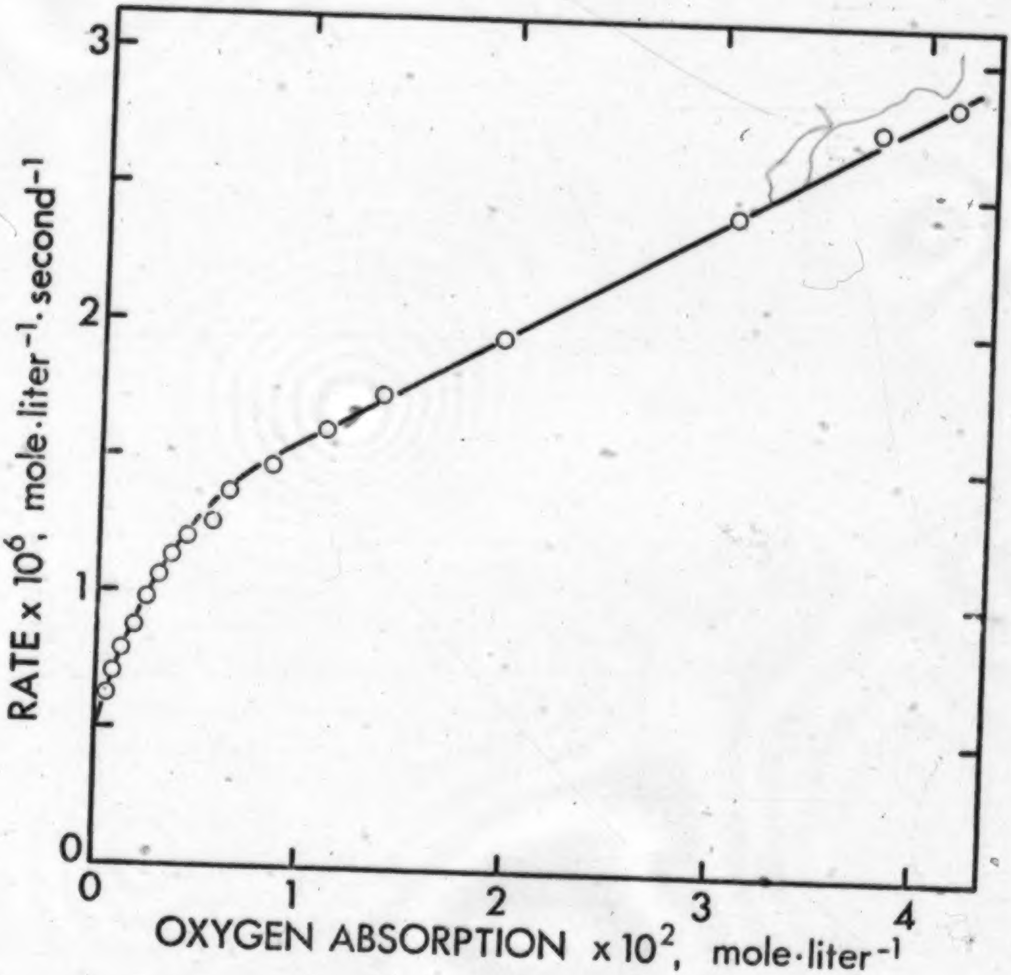


Figure 1. Rate of oxidation in relation to extent of oxidation. Autoxidation of ethyl linoleate at 55°C. and 46 mm Hg pressure of oxygen (redrawn from Bateman et al., 1953, p. 190).

factor other than the basic autoxidation mechanism which initiates the chain (see chain initiation, above).

This work in toto is the basis for the universally accepted mechanism of autoxidation. Experimentally, it has been shown that a double bond in a hydrocarbon chain activates a hydrogen on an adjacent C atom, and this effect increases with increasing unsaturation. Furthermore, numerous studies have confirmed that an induction period is typical of the initial stage of autoxidation, maximum O_2 uptake following only after maximum peroxide formation.

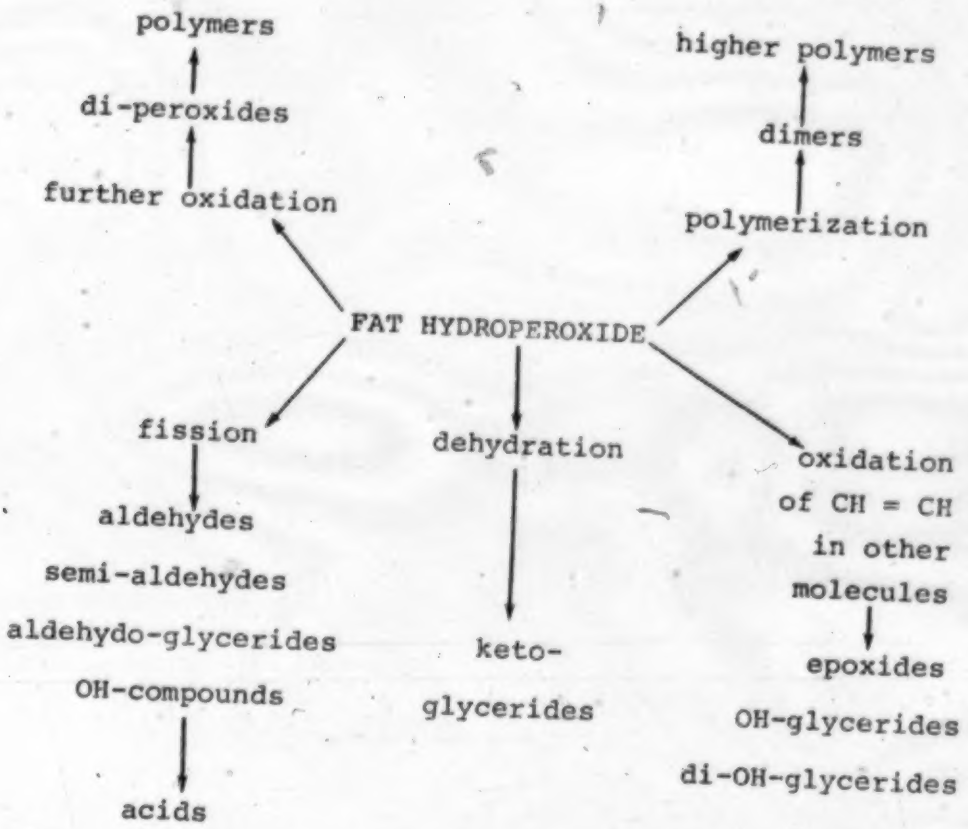
Hydroperoxides exist in equilibrium:

$$2\ ROOH \rightleftarrows (ROOH)_2$$

At low hydroperoxide concentration, the ratio of monomer to dimer is high and the chain reaction is initiated primarily by decomposition of the monomer. At high concentration, the ratio is reversed and chain initiation results mainly from dimer breakdown. The straight line portion of the curve in Figure 1 cannot be extrapolated to the origin because a portion of the autoxidation at all levels is initiated by decomposition of monomeric hydroperoxide.

2. Secondary Products.

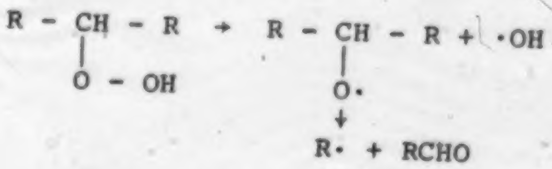
Autoxidation forms hydroperoxides first. They are non-volatile, tasteless, and odorless. Further oxidation is necessary before off-flavors are noticeable. Complicated types of products may be formed upon further decomposition:



The tendency for polymerization in food autoxidations is slight, except in frying operations where sustained heat induces formation of - O -, - O - O -, and - C - C - bridges, yielding a viscous mass eventually.

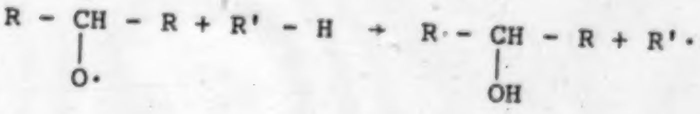
Typically, an autoxidation displays a characteristic induction period, in which oxygen absorption is nil. When any appreciable O_2 uptake begins, it is too late to avoid off-flavors, because of autocatalysis. At higher oxidation levels, direct 1,2- and/or 1,4- addition of oxygen may take place secondarily at the now conjugated diene structures of the fatty acid hydroperoxides, leading to the formation of various non-volatile monomeric oxygen-containing compounds (sometimes with carbonyl functions) and to dimers (Johnston et al., 1961).

The secondary oxidation products are generally odorless, but not tasteless. Assuming monomolecular homolytic cleavage (Bateman, 1954) we obtain from a hydroperoxide:



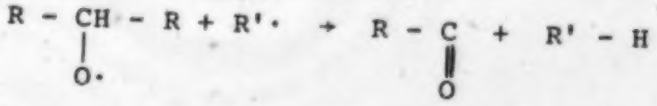
$$R-\underset{\displaystyle |\atop \displaystyle O-OH}{CH}-R \rightarrow R-\underset{\displaystyle |\atop \displaystyle O\cdot}{CH}-R+\cdot OH \qquad O\cdot \rightarrow R\cdot + RCHO$$

Abstraction of a hydrogen from another molecule can, e.g., result in a new free radical and an alcohol:



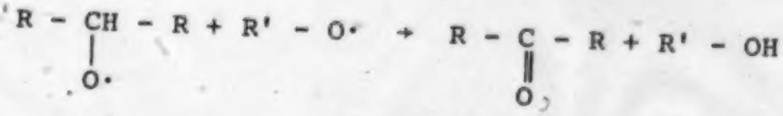
$$R-\underset{\displaystyle |\atop \displaystyle O\cdot}{CH}-R+R'-H \rightarrow R-\underset{\displaystyle |\atop \displaystyle OH}{CH}-R+R'\cdot$$

Interchange between two free radicals can lead to chain termination and ketone formation:



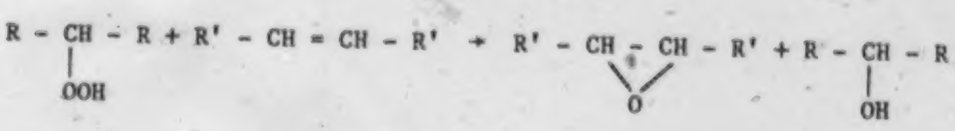
$$R-\underset{\displaystyle |\atop \displaystyle O\cdot}{CH}-R+R'\cdot \rightarrow R-\underset{\displaystyle \|\atop \displaystyle O}{C} + R'-H$$

alternatively,



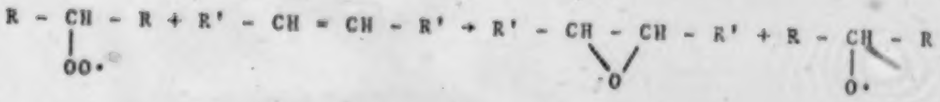
$$R-\underset{\displaystyle |\atop \displaystyle O\cdot}{CH}-R+R'-O\cdot \rightarrow R-\underset{\displaystyle \|\atop \displaystyle O}{C}-R+R'-OH$$

The formation of epoxides following reaction of hydroperoxides and/or their radicals with double bonds can also occur:



$$R-\underset{OOH}{\underset{|}{CH}}-R+R'-CH=CH-R' \rightarrow R'-\underset{\diagdown O \diagup}{CH-CH}-R'+R-\underset{OH}{\underset{|}{CH}}-R$$

and



$$R-\underset{OO\cdot}{\underset{|}{CH}}-R+R'-CH=CH-R' \rightarrow R'-\underset{\diagdown O \diagup}{CH-CH}-R'+R-\underset{O\cdot}{\underset{|}{CH}}-R$$

Most of the short-chain, volatile, monomeric products (predominantly carbonyls) have distinctive odor and taste. The non-volatile products can be monomeric (e.g., semi-aldehydes) or polymeric and are usually odorless and tasteless. The major part of the volatiles resulting from autoxidation of oils and fats of all kinds consists of aldehydes, but total volatiles are always less than 4% of the products. In practice, the aldehydes cause unacceptable flavor and aroma in foods containing lipids. Many aldehydes possess an organoleptic threshold in the dilution range of $1:10^9$ to $1:10^{10}$.

Table 1 gives a prospect of mono-hydroperoxides and mono-aldehydes which are formed by autoxidation of the most important unsaturated fatty acids. This is a conservative view, because only the most active methylene group is taken into account as the site of oxygen attack.

Table 1. Survey of the Hydroperoxides and Aldehydes Which May be Formed in the Autoxidation of Some Unsaturated Fatty Acids*

Fatty Acid	Methylene Group Involved	Isomeric Fatty Acid Hydroperoxides	Aldehydes
Oleic acid	11	11-hydroperoxy Δ^{9}-	C_8-al
		9-hydroperoxy Δ^{10}-	C_{10}-Δ^{2}-enal
	8	8-hydroperoxy Δ^{9}-	C_{11}-Δ^{2}-enal
		10-hydroperoxy Δ^{8}-	C_9-al
Linoleic acid	11	13-hydroperoxy $\Delta^{9,\ 11}$-	C_6-al
		11-hydroperoxy $\Delta^{9,\ 12}$-	C_8-Δ^{2}-enal
		9-hydroperoxy $\Delta^{10,\ 12}$-	C_{10}-$\Delta^{2,4}$-dienal
Linolenic acid	14	16-hydroperoxy $\Delta^{9,\ 12,\ 14}$-	C_3-al
		14-hydroperoxy $\Delta^{9,\ 12,\ 15}$-	C_5-Δ^{2}-enal
		12-hydroperoxy $\Delta^{9,\ 13,\ 15}$-	C_7-$\Delta^{2,4}$-dienal
	11	13-hydroperoxy $\Delta^{9,\ 11,\ 15}$-	C_6-Δ^{3}-enal
		11-hydroperoxy $\Delta^{9,\ 12,\ 15}$-	C_8-$\Delta^{2,5}$-dienal
		9-hydroperoxy $\Delta^{10,\ 12,\ 15}$-	C_{10}-$\Delta^{2,4,7}$-trienal
Arachidonic acid	13	15-hydroperoxy $\Delta^{5,\ [illegible],\ 11,\ 13}$-	C_6-al
		13-hydroperoxy $\Delta^{5,\ 8,\ 11,\ 14}$-	C_8-Δ^{2}-enal
		11-hydroperoxy $\Delta^{5,\ 8,\ 12,\ 14}$-	C_{10}-$\Delta^{2,4}$-dienal
	10	12-hydroperoxy $\Delta^{5,\ 8,\ 10,\ 14}$-	C_9-Δ^{3}-enal
		10-hydroperoxy $\Delta^{5,\ 8,\ 11,\ 14}$-	C_{11}-$\Delta^{2,5}$-dienal
		8-hydroperoxy $\Delta^{5,\ 9,\ 11,\ 14}$-	C_{13}-$\Delta^{2,4,7}$-trienal
	7	9-hydroperoxy $\Delta^{5,\ 7,\ 11,\ 14}$-	C_{12}-$\Delta^{3,6}$-dienal
		7-hydroperoxy $\Delta^{5,\ 8,\ 11,\ 14}$-	C_{14}-$\Delta^{2,5,8}$-trienal
		5-hydroperoxy $\Delta^{6,\ 8,\ 11,\ 14}$-	C_{16}-$\Delta^{2,4,7,10}$-tetraenal

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* From Badings (1960).

Numerous workers have isolated aldehydes from autoxidizing lipid systems in numbers often in excess of the theoretical. *Cis*,*trans* - isomerism and double bond shifts resulting from the tendency to form thermodynamically more stable compounds, as well as hydroperoxide formation at sites other than α-methylenic groups, have been offered as explanations.

Hydroperoxide formation as the major first step in autoxidation is well established for all time, but not all substrate is converted to hydroperoxide initially. Some reaction pathways leading from and around hydroperoxide have been established, but further knowledge is still needed to explain products which are being discovered continually by ever more refined analytical methods. Limited space allows only one example of many: during autoxidation of soybean oil, saturated hydrocarbons can arise early when aldehydes are either absent or not detectable (Selke *et al*., 1970).

3. Pro-oxidants.

The characteristic feature of lipid autoxidation is the ease with which it can be influenced by factors other than the usual ones of concentration of reactants and temperature. Accelerating factors are ultimately radical formers. Most important are light, ionizing radiation, peroxides and transition metal ions. Salt (NaCl) exhibits an accelerating effect on lipid oxidation in some food systems (such as sausage) but its role has not been proved. It is presumed to mobilize metal catalysts.

The mechanism of light in accelerating fat oxidation is known to be different from that of ionizing radiation, because the products of reaction are different, but no precise information is available. U.V. and short wave (blue) light probably function by photolysis of peroxides, yielding radicals which initiate new reaction chains. Deterioration in light differs again from the thermal reaction as determined, e.g.,by the off-flavors produced.

In the presence of heavy metals, such as Fe, Co, Cu, Mn, and their organic and inorganic salts - even in minute trace amounts - the breakdown of initially formed hydroperoxides is catalyzed. New radicals are formed which themselves initiate reaction chains with substrate or oxygen. According to Bawn (1953):

$$Me^{++} + ROOH \rightarrow Me^{+++} + RO\cdot + OH^-$$

$$Me^{+++} + OH^- \rightarrow Me^{++} + \cdot OH$$

An analogous set of reactions induced photochemically is given by Bolland and Gee (1946):

$$Me^{+++} + ROOH \rightarrow Me^{++} + ROO\cdot + H^+$$

$$ROO\cdot + RH \rightarrow ROOH + R\cdot$$

$$R\cdot + O_2 \rightarrow ROO\cdot \quad \text{etc.}$$

All theories concerning metal catalysis have one point in common: easily changed valence favors redox reactions (donation or acceptance of electrons) which, in their turn, tend to maintain certain concentrations of active radicals. The latter are strong oxidizing agents and initiate further reaction chains via Farmer's mechanism.

Pigments (lipochromes) and biocatalysts will be dealt with separately.

4. Antioxidants

Antioxidants are free radical scavengers and do not inhibit oxygen uptake directly. They absorb activation energy from excited fatty acid peroxide free radicals, thereby inactivating them.

The effectiveness of natural and synthetic antioxidants is determined by the presence of at least one phenolic OH group. If several OH groups are present, p-isomers are more effective than o-isomers, while m-isomers are weak, or non-effective antioxidants, or even prooxidants (Täufel and Rothe, 1951).

The chain-breaker effect of phenolic antioxidants is exerted at the stage of ROO· as follows (Bolland and ten Have, 1947):

$$\mathrm{ROO\cdot} + \mathrm{AH_2} \rightarrow \mathrm{ROOH} + \mathrm{AH\cdot}$$

$$2\ \mathrm{AH\cdot} \rightarrow \mathrm{AH_2} + \mathrm{A}$$

$$\mathrm{ROO\cdot} + \mathrm{AH\cdot} \rightarrow \mathrm{ROOH} + \mathrm{A}$$

where AH_2 is the primary antioxidant. The species AH· can be quite stable.

Synergists are compounds which enhance the ability of primary antioxidants to function without being able to interrupt autoxidation by themselves. Good synergists generally are di- or multi-basic organic or inorganic acids (ascorbic, phosphoric, citric, maleic, fumaric) and certain phospholipids (*e.g.*, lecithin). These compounds act as H donators, continually regenerating the H donated by the primary antioxidant. Thus, they extend the induction period at their own expense. They are also metal chelators.

Pezold (1969) offers an interesting hypothesis for reconciling apparently paradoxical behavior of antioxidants in autoxidizing systems. His mechanism is based upon his own observations and those of others. Briefly, they are:

1. Most plant oils contain antioxidative agents naturally in optimum concentration. Further addition of this same antioxidant shows no enhancement of effect.
2. In non-marine animal fats, there are only minute quantities of antioxidants. Addition of more of the same primary antioxidant is effective as increased antioxidant protection.
3. Synergists show a strong effect when added

to plant oils, because primary antioxidants are already present. They do not halt autoxidation of animal fats.

4. Plant oils freed of their natural antioxidants (by chromatography) are fundamentally the same as animal fats.
5. Tocopherol catalyzes the breakdown of fatty acid hydroperoxide *in vacuo* to free radicals.

He found that the process of autoxidation in the presence of antioxidants was a result of three different competing reactions:

1. normal autoxidation (o)
2. antioxidant-inhibited oxidation (a)
3. antioxidant-catalyzed oxidation, or pro-oxidative reaction (p)

A comparative investigation of reaction rates of the three competing reactions disclosed the following relationship of activation energies (AE):

$$AE_a < AE_o < AE_p$$

The pro-oxidative reaction runs considerably slower than the antioxidative reaction at low temperatures and only gains influence over the net reaction after a higher temperature is reached. The rate of the antioxidative reaction increases with increasing concentration of antioxidant as long as the reaction partners (peroxy radicals) are available in sufficient quantity. Above the optimum concentration antioxidant molecules are available for pro-oxidative reaction. The latter is seen as cleavage of stable peroxides with formation of free radicals. Thus, with enough excess of antioxidant the substance functions pro-oxidatively.

Powerful protection against lipid oxidation is afforded by water in complex foods of low moisture content. Optimal stability is afforded by a monolayer of water molecules. Below this level of hydration rapid oxidation ensues and, above it, hydrolysis and subsequent oxidative deterioration occur. The monolayer is bound by functional groups of proteins and carbohydrates. Water attached at these sites prevents adsorption of O_2 by its presence. It also coordinates trace metals, thus reducing their catalytic effect. Finally, water stabilizes hydroperoxides by H-bonding.

The literature contains many reports concerning pro- and antioxidative effects of various amino acids and ascorbic acid upon the oxidative reactions of food lipid systems. The data and hypotheses are inconclusive and outside the scope of this thesis.

B. Biocatalysis.

Lipid peroxidation catalysts exist in wide variety in biological systems. They include transition metals in different states of coordination. Hematin compounds and lipoxygenases are most prominent because they have greater catalytic activity than other known catalysts and have therefore been studied most.

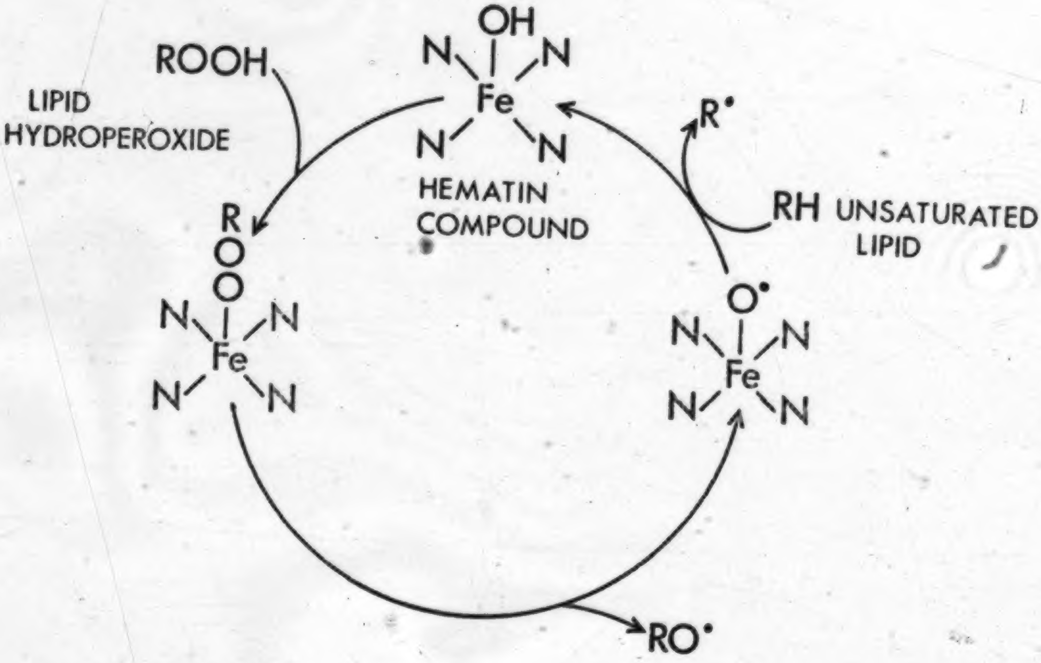
1. Hematin Compounds.

Lipid peroxidation catalyzed by hematin compounds is a basic deteriorative and pathological reaction and is becoming a subject of increasing research interest. Hematin catalyzed oxidative rancidity sets the ultimate limit to storage life of refrigerated and frozen meats.

All the hematin compounds occurring in nature catalyze oxidation of unsaturated lipids and other olefins. The products of hematin catalysis and autoxidation are similar, but comparison reveals that hematin catalysis has more rapid initiation and propagation reactions. In fact there may be no induction period, whereas autoxiation always displays an induction period.

Hematin catalysis is no more specific than autoxidation (with reference to substrate), but hematin-catalyzed oxidations of linoleate, *e.g.*, have activation energies comparable to lipoxygenase-catalyzed oxidations of linoleate, 3-6 kcal/mole; the activation energy of autoxidation of the same substrate is far more, ~ 15 kcal/mole (Tappel, 1961, p. 337).

Figure 2 is a schematic of hematin-catalysis. After reaction between hematin compound and lipid hydro-



$$RO\cdot + RH \longrightarrow ROH + R\cdot$$

$$R\cdot + O_2 \longrightarrow ROO\cdot$$

$$ROO\cdot + RH \longrightarrow ROOH + R\cdot$$

Figure 2. Proposed mechanism of hematin-catalyzed unsaturated lipid oxidation (redrawn from Tappel, 1961, p. 357).

peroxide - without valence change of the iron - the complex thus formed decomposes with homolytic scission at the - O - O - linkage to form two free radicals. As in autoxidation, many reactions are possible from these free radicals, depending on conditions. The main pathways in the presence of excess unsaturated lipid involve free radical chain reactions.

The evidence for regeneration of the hematin compound with each turn of the cycle is that small quantities of hematin catalysts cause the oxidation of large amounts of unsaturated lipids and the decomposition of large amounts of lipid peroxides.

During hematin catalyzed oxidation there is a concurrent oxidation of many labile compounds, including carotenoids, reducing enediols, polyphenols and the hematin catalyst itself.

Polyphenolic antioxidants function as chain breakers and their effective concentration is increased by synergists as discussed previously.

Inhibition by nitrogenous compounds, including cyanide, involves hemichrome formation and steric hindrance, preventing reaction with lipid hydroperoxide.

2. Lipoxygenases.

a. General Aspects

The enzyme lipoxygenase (linoleate: O_2 oxidoreductase, EC 1.13.1.13), formerly termed lipoxidase, occurs widely in plant tissues. The main sources are pulse vegetables and cereal grains. Other sources include potato, pumpkin, flaxseed, rapeseed and hemp. There is no evidence for its occurrence outside the plant kingdom. More than any other enzyme it necessitates the heat treatment in food processing known as blanching. It is therefore one of the most thoroughly documented enzymes pertinent in this field.

Lipoxygenase is a highly specific catalyst for the peroxidation of unsaturated fatty acids and their esters which contain the _cis_,_cis_-1,4-pentadiene system. This includes the essential fatty acids linoleic, linolenic and arachidonic, but not oleic.

Advance in knowledge concerning this enzyme came after the preparation of pure soybean lipoxygenase by Theorell _et al_., (1947). The principal initial product from soybean lipoxygenase-catalyzed oxidation of linoleic acid is

9 D - hydroperoxy - 10 - _trans_, 12 - _cis_ - octadecadienoic acid.
13 L - hydroperoxy - 9 - _trans_, 11 - _cis_ - octadecadienoic acid

is also formed, the ratio of these products varying with conditions and source of enzyme. The reaction is specific with respect to position of the methylene group of the _cis_,_cis_-1,4-pentadiene system and the point at which peroxidation occurs (Holman _et al_., 1969). For reaction to occur, the CH_2 group must be located at the 8th carbon atom as counted from the methyl end of the molecule (ω8-carbon). Peroxidation, then, will occur at the ω6 or at the ω10 carbon atom (i.e., at the 13- or 9- carbon atom, respectively) in the case of linoleic acid.

Variation at the carboxyl end of polyunsaturated fatty acids influence reactivity only in proportion to the influence on solubilization. Not only does solubility of a substrate fatty acid or its esters depend on pH, but so too could certain fundamental properties of the enzyme protein. Indeed pH profile of soybean lipoxygenase depends upon ionic strength of the medium. All factors which determine to what extent the hydrophilic enzyme has access to its hydrophobic substrate must be considered when estimating activity.

Polyphenolic antioxidants are the only important inhibitors of lipoxygenase. Nordihydroguaiaretic acid is the most potent inhibitor known. Although there is a lack of specificity among various antioxidant inhibitors, they are thought to function in like manner as competitive inhibitors of lipoxygenase by donating an active hydrogen for abstraction in place of the one donated by the substrate (see below). The chain breaker polyphenolic antioxidants could serve as criterion for identification of true lipoxygenases, provided proper precautions rule out non-enzymic catalysts. Cyanide, e.g., has no effect on lipoxygenase.

Theorell et al. (1947) reported that lipoxygenase did not require a metal or other prosthetic group. This conclusion was based upon the absorption spectrum of the enzyme and metal analysis. Although 0.3 atom of the Fe per mole of enzyme was detected, it was regarded as impurity. Chan (1973) reported that the Theorell enzyme contained 1 to 2 atoms of Fe per mole of enzyme (based upon atomic absorption spectroscopy) and that various chelating agents inactivate the enzyme. Pistorius and Axelrod (1974) established that lipoxygenase contains one atom of Fe per mole, that it is essential for enzyme activity, and that the Fe is present in the ferric state.

Tappel (1963) proposed a mechanism for lipoxygenase catalysis which is still generally accepted, although new knowledge continues to emerge. The latter can be considered to be supplementary (see below) but, by and large, substantiating Tappel's view. Briefly, it is:

> The enzyme protein acts as an electron sink, holding momentarily an electron from the active methylene group of the reactive unsaturated fatty acid and thus allowing oxidation to proceed. The five steps are:
>
> 1. Formation of an enzyme-substrate complex.
> 2. Attachment of O_2 to the enzyme in proximity to the substrate.

3. Formation of a free radical at the methylene group by removal of a hydrogen atom. The enzyme protein holds an electron momentarily from the alpha methylene group, thus allowing oxidation. The hydrogen ion goes to the medium or directly to the protein.
4. Isomerization of double bonds. When the electron moves to conjugate the double bonds, the diradical oxygen attacks the formed free radical, resulting in the peroxy radical.
5. A molecule of hydroperoxide is formed, after picking up a hydrogen ion, and then released.

Mitsuda _et_ _al_. (1967a) produced evidence indicating that methionine residues accept the H (step 3 above) and, also, that there is a hydrophobic binding site near the active center.

Christopher _et_ _al_. (1970) isolated two isoenzymes of soybean lipoxygenase which had differences in substrate specificity, heat stability, turnover number and activation by Ca^{2+} ion. One of these, lipoxygenase-1, is inhibited by Ca^{2+}. The other, lipoxygenase-2, is activated by Ca^{2+}, a process which the authors characterize as overcoming substrate inhibition. The second is the one which is more heat labile. Each of them possesses a molecular weight of 102,000 as determined by chromatography on Sephadex G 150. Yamamoto et al. (1970) isolated these two isoenzyes also and named the lipoxygenases a and b, respectively. They confirmed the Ca^{2+} inhibition and activation. Numerous other reports exist in the literature of isoenzymes of lipoxygenases.

Restrepo et al. (1973) attempted to elucidate the peculiar importance of sequence of addition of the activating Ca^{2+}. When added to the reaction mixture prior to or concurrently with lipoxidase, activation will occur, but it will not if added after the enzyme. They established that adsorption on glass surfaces is not prevented by Ca^{2+}. They ruled out the possibility that phytates may be involved in activation or inhibition by Ca^{2+}. They established that no Ca^{2+} activation occurs if trilinolein is used as substrate, and that Ca^{2+} concentration affording the maximum activation is independent of lipoxygenase concentration, but varies with linoleic acid concentration when lipoxygenase is constant. In the latter case, it is about equimolar with linoleic acid concentration. However, they did not have an explanation for this behavior. It is evident that research on these enzymes has fundamental problems yet to solve. Because of their importance, work in many laboratories is continuing. The near future may reveal more complete maps of active centers which presumably could elucidate substrate preference and versatility among isoenzymes.

b. Pea Lipoxygenase

Peas are an important part of the diet of most peoples of the earth. Research and application of its results constitute an important input of the pea processing industry, which has been evolving since the late nineteenth century. Since the advent of frozen foods, scientific study has received much impetus, because peas are the vegetable of greatest economic importance in frozen preservation.

Present knowledge of the role of enzymes in deterioration of flavor and nutritive value is incomplete. In the past, emphasis has been given to the presence or absence of catalase and peroxidase (hematin compounds) in the processed product, but the importance of lipoxygenase and lipase as specific catalysts of lipid breakdown is now recognized (Wagenknecht and Lee, 1958). The destruction of the latter could provide a better criterion for control purposes.

In 1970, Eriksson and Svensson isolated and purified two active lipoxygenase isoenzymes from peas and reported molecular weights of 72,000 and 67,000 on the basis of amino acid analysis and ultracentrifugation. These probably corresponded to the two isoenzymes of lipoxygenase detected in the pea by Hale _et al_. (1969), who separated lipoxygenase into bands with gel electrophoresis and visualized them with acidic KI staining. Haydar and Hadziyev (1973) reported an entity of lipoxygenase which had a molecular weight of 74,000 by gel electrophoresis. They found three to four isoenzymes by the zymogram method. The same group (Haydar et al. 1974) isolated a pea lipoxygenase, which possessed only one active isoenzyme, in the presence of the endogenous Ca^{2+} ion of the pea. The enzyme activity was found to be lower than when Ca^{2+} interference was avoided. Weber, Arens and Grosch (1973) separated pea lipoxygenase into two peaks with DEAE-cellulose column chromatography, and Anstis and Friend (1974) separated five active isoenzyme fractions of lipoxygenase from dwarf peals using a carboxymethyl-cellulose column.

Unblanched (frozen and dried) green peas can develop off-flavors from accumulating volatile aldehydes which result from lipoxygenase catalyzed oxidation of linoleic and linolenic acid, the major polyunsaturated fatty acids in the pea. Recently, Leu (1974a) examined primary oxidation products of this reaction using gas chromatography. He found equal amounts of the 9- and 13- hydroperoxides of linoleic acid. Leu (1974b) analyzed volatile secondary products, also, and found aliphatic saturated and unsaturated n-aldehydes. His findings show that the amounts and ratios of primary hydroperoxide isomers vary with conditions and enzyme source, but that most of the volatiles derive from these primary products. Furthermore, enzymes from different sources yield the same aldehydes in similar ratios, although the ratios of isomeric primary products differ. In summary, his results are compatible with existing theories concerning formation of secondary products. Homologous aldehydes are formed one from another by successive rupture of one carbon bond in the chain (n-hexanal, n-pentanal, n-butanal, n-propanal, ethanal; the quantities of each diminish in the order given). Also, hydrocarbons and furan derivatives were observed.

C. Pigment Destruction.

The three major classes of natural pigments,

- the anthocyanins and flavones,
- the chlorophylls and
- the carotenoids

are increasingly unstable in the order listed. That is, they are subject to oxidations and isomerizations, with subsequent loss of pigmentation. Only the last two groups are to be considered here in connection with peas. Acceptability of any pea pack depends upon the chlorophyll-pheophytin ratio or the ratio of the attractive fresh green of the former to the olive green of the latter (a chlorophyll heat-degradation product). Carotenoids, chiefly β-carotene, are of nutritional significance as potential pro-vitamin A source.

Although autoxidations and photoxidations of isolated pigments have been carried out under laboratory conditions, it is generally accepted by food chemists that pigment destruction occurring in food products is coupled with lipid oxidation and that a free radical mechanism is involved.

Strain (1941) observed that chlorophyll was oxidized in a system containing an aqueous soybean extract, a fat and oxygen. In 1955, Mapson and Moustafa reported that chlorophyll was bleached during the oxidation of glutathione by a pea extract in the presence of linoleic acid. The oxidation of glutathione was attributed to the lipoxygenase catalyzed oxidation of linoleic acid. Blanched frozen peas show no loss of chlorophyll, but it is degraded in frozen raw peas. Wagenknecht and Lee (1958) added various enzyme preparations to enzymatically inactive peas and discovered that lipase and lipoxygenase together, lipase alone, and lipoxygenase alone catalyzed 35%, 28% and 12% destruction of chlorophyll, respectively.

Holden (1965) tested several legume seed extracts for chlorophyll beaching activity and their response to addition of fatty acids. Linoleic and linolenic acids showed the greatest bleaching rates in the presence of soybean extract. Neither peroxidized linoleic acid nor purified lipoxygenase plus lipid bleached the chlorophyll as rapidly as the crude extracts. Therefore, she postulated a co-oxidation factor for chlorophyll loss. These observations were supported by Buckle and Edwards (1970) in studies of frozen soybeans and peas. They noted a pH optimum for chlorophyll bleaching lower than that required for lipoxygnase and a requirement for a crude extract component besides lipoxygenase which was a heat-labile factor. This factor could be isolated by $(NH_4)_2SO_4$ precipitation. A similar co-oxidation factor was postulated by Kies et al. (1969) in the coupled oxidation of carotene.

Zimmerman and Vick (1970) demonstrated that chlorophyll is bleached only in the presence of linoleic acid, lipoxygenase and hydroperoxide isomerase. It is proposed that the enediol which proceeds from the action of the enzyme, hydroperoxide isomerase, upon the primary product of lipoxygenase catalysis,

$$\text{fatty acid hydroperoxide} \rightarrow \alpha\text{-ketol} \rightleftarrows \text{enediol},$$

attacks the conjugated double bond systems of pigment molecules, yielding colorless compounds not yet identified.

Orthoefer and Dugan (1973) reported that the usual lipoxygenase inhibitors also inhibit chlorophyll bleaching in model systems with linoleic acid, chlorophyll, and lipoxygenase. They claimed to have improved upon Holden's chlorophyll assay technique and concluded that lipoxygenase is the only enzyme required in the system. They explain the lower pH optima reported by others on the basis of alterations in vitro of critical micelle concentration of lipid substrate during the assay of bleaching (maximum pigment destruction occurs at the critical micelle concentration). The authors conclude that chlorophyll bleaching is mediated through coupled oxidation with the unsaturated lipid substrate, since the pigment is not bleached with previously oxidized fatty acid.

Blain (1970) stated in his paper that carotene bleaching activity in general could be more readily attributed to intrinsic hematins than lipoxygenases, although it is "generally assumed" that this activity is due to lipoxygenase. Chou and Breene (1972) conclude that decoloration of β-carotene is an autoxidative reaction when lipid is excluded from the system and that the presence of either water or an antioxidant inhibits the rate of decoloration in a manner similar to that for lipid oxidation.

Considerable research on wheat lipoxygenase and carotene oxidation has shown the importance of lipoxygenase action during dough mixing and its co-oxidation of carotene (Irvine, 1959). Carotene co-oxidation can be controlled by selection of wheat of suitable lipoxygenase and carotene contents, by addition of legume flours containing the enzyme during dough mixing and by controlled aeration during mixing. In contrast, durum wheat products such as semolina, macaroni and spaghetti are selected on the basis of minimal lipoxygenase activity to preserve the carotenoid pigmentation. Soybean lecithin is even added to synergize natural primary antioxidants.

Coupled oxidation of carotenoids in lipoxygenase reaction is complex both with respect to kinetics and products formed. Tookey et al. (1958) demonstrated that free radical intermediates of the linoleate - lipoxygenase reaction cause hydrogen abstractions which are relatively non-specific and which are capable of oxidizing carotene. Another line of evidence for the existence of the free radical intermediate is provided by Fridovich and Handler (1961). Concurrent oxidation of other carotenoids, oil soluble vitamins, essential oils, polyphenolic compounds such as flavonols (cf. Heimann and Reiff, 1953) and various antioxidants can occur via abstraction of their hydrogens by the unsaturated fatty acid peroxy free radical intermediate which is crucial to the lipoxygenase reaction mechanism. Coupled oxidations can also arise by other free radical mechanisms previously discussed.

D. Photoxidation

It is a matter of general experience in food processing and storage that the most favorable conditions for maintaining original β-carotene content are also those which favor shelf life of fats, viz., exclusion of oxygen, inclusion of antioxidants, and exclusion of light. It is also common knowledge that yellow-pigmented fats, such as beef tallow and butter, bleach out during storage, especially if acid number is appreciable and light exposure is not avoided. The nutritive value of alfalfa can be seriously impaired during harvesting and storage, because of the coupled oxidation of carotene. Thus, quick drying is superior to field drying in the sun where chlorophyll has an opportunity to function as photosensitizer in the oxidation.

The utilization of electromagnetic energy in photoxidations depends on the presence of a chromophore in the substrate or upon the presence of a photosensitizer. A photoactivated molecule re-emits its energy either as heat or as electromagnetic energy of lower wavelength. It transfers its energy to the substrate or to oxygen, resulting in photoxidation. The presence of a photosensitizer may have a profound effect on rate of oxidation and the products formed.

Photoactivated intermediates possess more energy than those normally involved in autoxidation. Khan _et_ _al_. (1954) found that photoxidation of 1,4-dienes yielded only 1- and 5-hydroperoxides with inversion of configuration at one double bond. When chlorophyll was introduced an appreciable proportion of 3-hydroperoxide was also formed. Furthermore, whereas without chlorophyll _cis_,_trans_ hydroperoxides result, both double bonds were inverted to form _trans_,_trans_ hydroperoxide in the presence of the photosensitizer.

According to Scott (1965, p.95 ff.) photoxidation involves the same chain propagating steps as autoxidation occurring in the dark. The difference lies in the nature of the initiating step and in the rate of achievement of the linear part of the oxidation curve. That is, light accelerates the rate of approach to the steady state of oxidation.

Thus, the energy supplied by exposure to light is an important factor in the initiation of oxidative processes in food fats. A test designed to exploit this principle has been developed to determine stability of edible oils (Moser _et_ _al_., 1965). The development of surface oxidation in butter during cold storage under bright lights has been established (de Man _et_ _al_., 1965).

The question as to the role of β-carotene in oxidizing fats and oils has been discussed for some time. Whether or not this pigment acts pro-oxidatively or anti-oxidatively depends, apparently, upon the presence or absence of light.

Chevallier _et_ _al_. (1948) reported that carotene inhibits autoxidation of triolein in the dark (at 40°) but accelerates the oxidation in monochromatic U.V. light having a wavelength of 3400 Å. These authors did not carry the experiments beyond the point of total carotene destruction. Probably the pigment acts as a photosensitizer during photoxidation, thereby furthering the formation of free radicals. Lee (1955) reported that peroxide values of carotene-oil mixtures exposed to sunlight are about equal to or slightly higher than controls not containing carotene. His results could be more informative had he measured O_2 uptake, since peroxides do not accumulate beyond a certain steady-state level.

Täufel et al. (1959) reported that chlorophyll exerts a pro-oxidative effect only in daylight. In the dark it has no effect on the autoxidation of unsaturated fats, unless phenolic antioxidants are added, in which case it is synergistic.

Lundberg (1962, p.41 ff.), studied the oxidation of methyl linoleate in the presence of chlorophyll and monochromatic light of wavelength 660 nm at 37°. He found that hydroperoxides were the primary products of oxidation and that the quantity formed was directly proportional to the amount of light absorbed. The mechanism is presumed different from that of ordinary autoxidation, because significant quantities of unconjugated hydroperoxide were formed and because α-tocopherol is ineffective as an inhibitor, indicating that a chain reaction does not take place.

Haydar (1974) observed that β-carotene exerted an antioxidant effect upon the course of photoxidation of butter fat during the early stages. After the β-carotene had been totally destroyed, the reaction accelerated significantly. He concluded that decomposition of β-carotene protects the fat in the early stages, but that breakdown products accelerate photxidation once all the pigment is exhausted. Friend (1958) reported that these breakdown products are hydroxylated to a certain degree. With this in mind, it seems reasonable to suppose that they could function as chain breaking phenolic antioxidants until they were no longer able to donate active hydrogens. At that stage the induction period would be over and the reaction would accelerate.

Haydar (1974) found, moreover, that in the dark β-carotene in low concentration seemed to be a pro-oxidant, and, at a five-fold increase in concentration, the pigment exerted an antioxidant effect. I suggest that this would have been shown to be an induction period had the experiment been extended to the point where β-carotene was depleted. From this point of view, the findings are reconciled with those of Friend, because at the low concentration (30 μg per gram butter fat) insignificant amounts of OH-compounds were formed.

E. Non-enzymic Oxidation of Pea Lipids in Model Systems.

Braddock and Kesterson (1974) have recently reported that carotenoids (including carotene) of citrus flavedo are well preserved for animal feeding stuffs only if treated with antioxidants or freezing. The potential of this important citrus by-product as a valuable source of pro-vitamin A in animal feeds is not fully realized at present, because the technology of drying has not overcome the problem of carotene loss. Many operators find that it does not pay to take such pains as are required to prevent browning. This problem, however, does not exist with alfalfa. Surely, moisture content in the finished product is important. According to Lea (1958) water promotes non-enzymic browning, which can result in the formation of antioxidant compounds. Although the dried flavedo is higher in moisture content than dried alfalfa, it is probably poorer in natural antioxidant content, and one is inclined to assign an important role to other plant constituents. For instance, the flavedo is very high in pectins.

According to Karel (1973) oxidizing lipids in the presence of proteins tend to interact by forming lipid-protein complexes. In most complexes studied, the lipid constituent contains phospho-lipid. The interaction usually involves electrostatic forces or salt bridges via divalent metals. They can lead to colored or insoluble compounds and even jeopardize the food value of the reactants.

Crawford _et al_. (1967) stated that lipid peroxidation products react with proteins in a number of ways. Malonaldehyde,

$$OHC - CH_2 - CHO$$

is a product of hydroperoxides of linolenic, arachidonic and other oxidized fatty acids. It can react irreversibly with amino acid residues.

Zirlin and Karel (1969) studied gelatin-linoleate interactions in the dry state and concluded that reaction of the two components can lead to scission of the protein as well as protein-protein crosslinking. Free radicals may form on α-carbons of the proteins and cysteyl radicals may form in proteins containing cysteine or cystine. Sulfur-containing proteins are more likely to crosslink than non-sulfur proteins, such as gelatin.

Free radical participation in lipoprotein formation, reactions and properties is a challenging subject of research being pursued currently at several institutions. At the University of California, Roubal (1970) interpreted the ESR signal obtained from proteins exposed to oxidized lipids to be caused by protein-lipid peroxide complexes.

Haydar and Hadziyev (1973) studied oxidation of pea lipids on carbohydrate and protein matrices in freeze-dried model systems. They concluded that the polarity of the lipid was more decisive in determining susceptibility to oxidation than the degree of unsaturation. On all matrices the polar lipids were more susceptible to oxidation, but particularly more so on pectin. This seems to be consistent with the findings of Braddock and Kesterson

(1974) concerning carotenoid destruction in flavedo. Compared to cellulose controls, albumins and globulins seemed to exert an antioxidative influence on neutral lipids, whereas albumin alone was antioxidative toward polar lipids. On the other hand, the globulins matrix accelerated polar lipid oxidation significantly. By way of critique concerning the authors' interpretation of their findings, I submit that before the cellulose matrix readings are taken at face value, the presence of catalytic cellulose autoxidation products must be ruled out (cf. Scott, 1965, p.320).

F. Methods of Assay.

The literature abounds in methods for measuring enzymic and non-enzymic lipid oxidation. Some are designed to measure products of oxidation and others, the consumption of oxygen or substrate. The latter are by far superior for most work, unless one is satisfied merely to demonstrate the existence of a certain product, or unless convenience dictates the choice of a less accurate approach.

The reader is referred to Tappel (1962) for manometric and spectrophotometric methods of assaying lipoxygenase, to Mitsuda et al. (1967b) for a method of measuring the activity of this enzyme by polarography, and to de Vore and Solberg (1974) for a description of the use of the differential respirometer in measuring oxygen uptake.

III. EXPERIMENTAL

Materials and Chemicals.

Wrinkled pea seeds (Pisum sativum L., var. Homesteader), air dried, were obtained from Seed Centre Ltd. (Edmonton, Alberta). The seeds were stored at a relative humidity of 20% and 15°C. for five months. Thereafter, they were kept at 4°C. in a storage cold room. After one year, germination testing revealed viability of better than 90%. Chlorophyll a, chlorophyll b, and total carotenoid contents were determined to be 0.0152, 0.206 and 0.0046 mg per gram on a dry weight basis, respectively (moisture content 15.93%).* Isolation of lipoxygenase, albumins and globulins, from these peas is discussed under Methods.

Wheat protein fractions, which were used for matrices in preliminary studies of non-enzymic oxidation of linoleic acid, were kindly provided by Dr. D. Hadziyev. They represented surplus material from previous research (Skura, 1972, p.32). Triticum vulgare L., vars. Park and Thatcher, obtained from the Alberta Wheat Pool and graded as foundation No.1, were each extracted for three different fractions. These were glutenins (gluten protein soluble in 0.05 N acetic acid), globulins (soluble in 0.4 M NaCl), and gliadins (soluble in 70% (w/w) ethanol). After extraction they were freeze-dried and stored in dry, air-tight containers as pure white powders.

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* Chlorophyll concentrations were determined with the equations and technique given by Maclachian and Zalik (1963) and carotenoids by the equation of von Wettstein (1957), cited by Maclachlan and Zalik, whereby measurement of absorption is made of the pigments in acetone solution at the appropriate wavelengths.

Linoleic acid, obtained from Fisher Scientific Co. (Fair Lawn, N.J.), was freshly distilled before use at 149-150°C. under a pressure of 0.4 mm Hg. β-Carotene, obtained from ICN Pharmaceuticals Inc. (Cleveland, Ohio), was recrystallized from benzene-methanol.

Linoleic acid, its methyl ester, and trilinolein, all of greater than 99% purity, which were used in substrate testing of the enzyme, were obtained from Hormel Institute (Austin, Minn.).

A 1.7 gram-package of commercial Spanish saffron (McCormick) was obtained from a local grocery store. This commodity, consisting of stigmas of Crocus sativus L., was used for the extraction of crocin, the digentiobiose ester of crocetin, an acid carotenoid.

Potato amylose and soybean lipoxygenase (EC 1.13.1.13)* were purchased from Sigma Co. (St. Louis, Mo.), Tween-20, -80 and Darco Activated Carbon Grade KB from Atlas Powder Co. (Brantford, Ont.), DEAE-cellulose from Baker Chem. Co. (Phillipsburg, N.J.), Bovine Serum Albumin (BSA) and dithiothreitol from Calbiochem (San Diego,

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* The supplier claims an enzyme activity of 156,460 units $\cdot mg^{-1}$, where one unit will cause an increase in A_{234} of 0.001 per min at pH 9.0 and 25°C. when linoleic acid is the substrate.

Calif.), acrylamide, and N-methylene-bis-acrylamide, from BioRad Labs. (Richmond, Calif.), TEMED and 2-mercaptoethanol from Eastman Organic Chem. (Rochester, N.Y.), pectin (geno-pectin slow set) from Food Products Ltd. (Montreal, Que.), amylopectin from ICN Pharmaceuticals Inc. (Cleveland, Ohio), Folin-Ciocalteu phenol reagent from BDH Lab. Chem. Div. (Toronto, Ont.), Sephadex G 150 from Pharmacia (Uppsala, Sweden), silicic acid for chromatography, 100 mesh, from Mallinckrodt (New York, N.Y.), and SDS, Tris and other chemicals, all of reagent grade, were obtained from Fisher Scientific Co. (Fair Lawn, N.J.). Certified protein standards used for molecular weight determinations, cytochrome c, chymotrypsinogen A, ovalbumin, BSA and human globulin (mol. wt. 12,000; 25,000; 45,000; 67,000 and 160,000, respectively) and Coomassie Brilliant Blue stain were obtained from Schwarz/Mann Co. (Orangeburg, N.Y.). Cardiac tropomyosin (mol. wt. 36,000) was kindly provided by Dr. F.H. Wolfe of this Department. Whatman No.3 filter paper, a thick, medium speed paper with high retention, was used to cut circles of 3.0 cm diameter for matrix studies.

Equipment.

Centrifuges used were: 1) Sorvall SS-1 Superspeed Angle Centrifuge manufactured by Ivan Sorvall Co. Inc. (Norwalk, Conn.) and 2) Spinco LC 2-65 B Ultracentrifuge with rotor type 40, Beckman Instr. Inc., Spinco Div. (Palo Alto, Calif.). Electrophoresis equipment was from Buchler Instruments (Fort Lee, N.J.). The electrolytic destainer was from Canalco (Rockville, Md.). Oxygen consumption measurements were performed with: 1) Gilson differential respirometer Models GR 20 and GP 8 (Gilson Medical Electronics Inc., Middleton, Wisc.) set to oscillate 104 times per minute with a 3.5 cm stroke, 2) a Biological Oxygen Monitor (also referred to as the oxygraph), Model 53, equipped with a Clark electrode (Yellow Springs Instruments, Yellow Springs, O.) connected to a Beckman 100 mV potentiometric recorder (Beckman Instr. Inc., Fullerton, Calif.) and 3) a Laboratory Oxygen Analyzer, Model 777 (Beckman Instr. Inc.). Supplementary illumination was provided by BLAK-RAY long wave UV, Model X-4 (Ultra-Violet Products, Inc., San Gabriel, Calif.). All spectrophotometric determinations were performed with Unicam SP 1800 UV recording spectrophotometer (Pye Unicam Ltd., Cambridge, England). The fraction collector used was the ISCO Model 327 (Instruments Specialties Co. Inc., Lincoln, Nebr.). Freeze-drying was done with the RePP instrument, manufactured by the VirTis Co. Inc., (Gardiner, N.Y.). The peristaltic pump employed in column chromatography was the LKB Perspex with silicone rubber tubing of 1.1 mm i.d. (LKB Produkter, AB, Bromma, Sweden). Emulsions were enhanced with a Raytheon sonicator, 250 W, 10 kc sonic oscillator (Raytheon Manuf. Co., Waltham, Mass.). Biological Oxygen Monitor and spectrophotometer cuvette temperatures were maintained with Model 154 Lo-Temptrol circulating baths, filled with water (Precision Scientific Co., Chicago, Ill.). Gel scanning was performed by Chromoscan MK II double beam recording and integrating densitometer at 620 nm, gear ratio 1:3 (Joyce, Loebl and Co. Inc., Goteshead, England). Heating of enzyme solutions up to 20 min. at specified temperatures was accomplished with a Tecam Dri-Blok DB-3 solid state heating block (Techne Cambridge Ltd., Cambridge, England).

Methods.

A. Lipoxygenase.

1. Isolation and Purification

Enzyme preparations were extracted from non-defatted pea seeds, because defatting yielded consistently lower activity.

One enzyme batch was prepared as follows (Procedure I). A portion of 100 g pea seeds was ground, with dry ice pellets, to a powder in a Waring blendor. The powder was extracted by stirring with 1.0 liter of 0.1 M Tris-Cl buffer, pH 7.2, for 16 h. The slurry obtained was filtered through six layers of cheesecloth and centrifuged at 20,000 x g for 15 min. The supernatant was centrifuged at 140,000 x g for 2.5 h. This supernatant was then precipitated with 25% ammonium sulfate saturation for 30 min, the precipitate discarded, and the supernatant again precipitated with ammonium sulfate, but at 50% saturation. The precipitate was collected, dissolved in 90 ml of 50 mM Tris-Cl buffer, pH 7.2, and dialyzed against 40 volumes of the same buffer for 15 h with several changes of buffer. The dialyzate was further purified by Sephadex and DEAE-cellulose column chromatography.

Sephadex G 150, allowed previously to swell in 0.02% azide solution, was poured into a 1.5 x 90 cm glass column and equilibrated with 50 mM Tris-Cl buffer, pH 7.2. An aliquot of 6.7 ml of dialyzate, containing 400 A_{280} units, was applied to the column and eluted with the same buffer at a constant rate with the LKB pump. Fractions of 4.5 ml were collected every 15 min. The A_{280} of each fraction was determined, all those displaying absorbance being then tested for lipoxygenase activity. The fractions containing lipoxygenase were combined and then the enzyme was precipitated by 50% ammonium sulfate saturation. After centrifugation (20 min at 15,000 x g) the pellet was dissolved in 10 mM Tris-Cl buffer, pH 6.5, containing 2 mM $CaCl_2$ and 1 mM 2-mercaptoethanol (Tris-Ca^{2+}-SH). The solution was then dialyzed against 40 volumes of the same buffer for 5, 10 or 15 h with several changes.

The DEAE-cellulose column (2.5 x 45 cm) was equilibrated with Tris-Ca^{2+}-SH buffer. Amounts of 30 to 120 A_{280} units of the dialyzed lipoxygenase were applied to the column and elution was performed with the same buffer combined with a shallow linear NaCl gradient ranging from 0.0 to 0.2 M. A Zeiss Abbe' refractometer was used to monitor the gradient. Fractions of 4.5 ml were collected every 12 min and assayed for A_{280} and lipoxygenase activity.

A second, similar method (Procedure II) omitted the Sephadex G 150 filtration step and the enzyme extraction was achieved in 10 mM sodium phosphate buffer, pH 7.0, for 16 h. After cheesecloth filtration, low-and high speed centrifugation and ammonium sulfate precipitation as before, the pellet was dissolved in 90 ml of 10 mM sodium phosphate buffer, pH 7.0, and dialyzed against 40 volumes of the same buffer, with several changes, for 15 h. Amounts of 30 to 120 A_{280} units of dialyzate were chromatographed on the DEAE-cellulose column, as above. That is, the phosphate buffer, combined with the shallow salt gradient, was used for elution.

In a third approach (Procedure III) employing the two chromatographic steps, the precipitations with 25% and 50% ammonium sulfate were omitted. After extraction with 10 mM phosphate buffer, pH 7.0, for 16 h, low and high speed centrifugations, the clarified pea seed extract (140 A_{280} units) was applied to the Sephadex G 50 column. After elution with the same buffer, an aliquot of effluent was applied to the DEAE-cellulose column, and the lipoxygenase enzyme collected as before (cf. Figures 3,4 and 5).

All isolation and purification steps described were carried out in the cold (4°C.).

Protein determinations were done according to Lowry _et al_. (1951), as modified by Oyama and Eagle (1956), with crystalline BSA as standard (cf. Figure A1).

2. Characterization.

a. Lipoxygenase Activity

The oxygraph, equilibrated to the desired temperature, was used to measure the rate of oxygen consumption by a defined, buffered linoleic acid substrate containing an emulsifier. Our standard assay for lipoxygenase activity is based upon a total reaction mixture volume of 3.0 ml, containing 7.5×10^{-3} _M_ linoleic acid, 0.25% Tween-20 and 0.1 _M_ Tris-Cl buffer, pH 7.0. The addition of, say 30 µl of a solution of enzyme introduces a 1% error into these figures and is ignored (cf. Derivation of Formula for Converting Oxygraph Readings to Absolute Values, and Figure A2).

For the carotene bleaching experiments, the method of Ben Aziz _et al_. (1971) was adapted to the oxygraph with the modifications being an increase in quantities

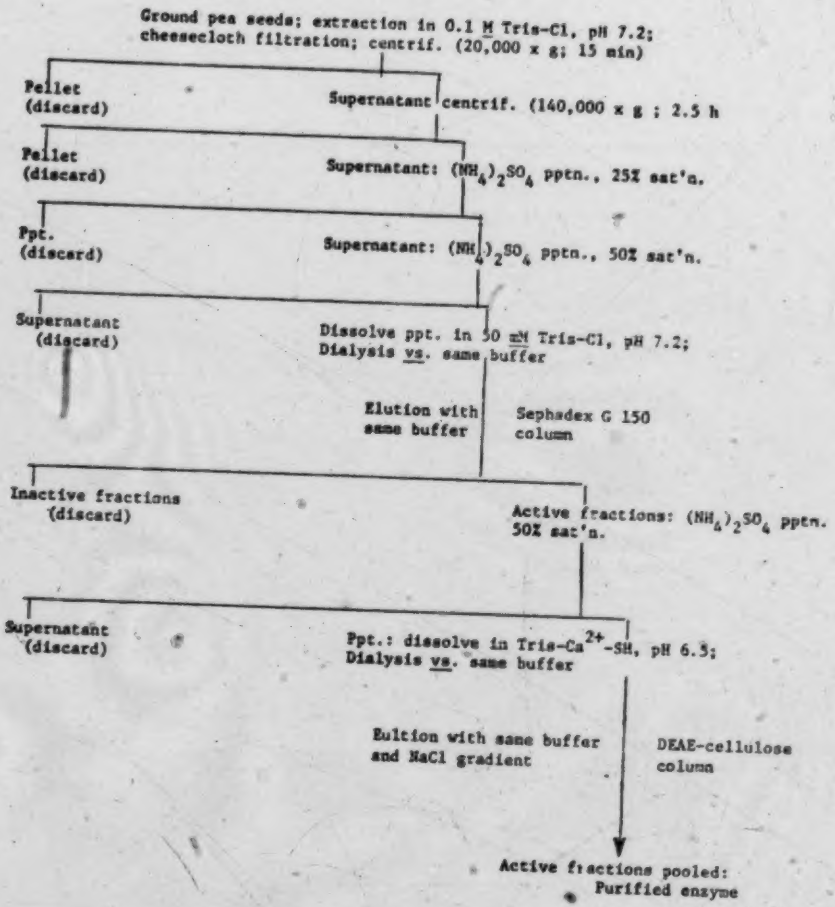

Ground pea seeds; extraction in 0.1 M Tris-Cl, pH 7.2; cheesecloth filtration; centrif. (20,000 x g; 15 min)
Pellet (discard)
Supernatant centrif. (140,000 x g ; 2.5 h
Pellet (discard)
Supernatant: $(NH_4)_2SO_4$ pptn., 25% sat'n.
Ppt. (discard)
Supernatant: $(NH_4)_2SO_4$ pptn., 50% sat'n.
Supernatant (discard)
Dissolve ppt. in 50 mM Tris-Cl, pH 7.2; Dialysis vs. same buffer
Elution with same buffer
Sephadex G 150 column
Inactive fractions (discard)
Active fractions: $(NH_4)_2SO_4$ pptn. 50% sat'n.
Supernatant (discard)
Ppt.: dissolve in Tris-Ca^{2+}-SH, pH 6.5; Dialysis vs. same buffer
Eultion with same buffer and NaCl gradient
DEAE-cellulose column
Active fractions pooled: Purified enzyme

Figure 3. Flow diagram for pea lipoxygenase isolation by Procedure 1. Variations (Procedures II and III), in which certain steps were omitted and different buffers were employed, are described in the text.

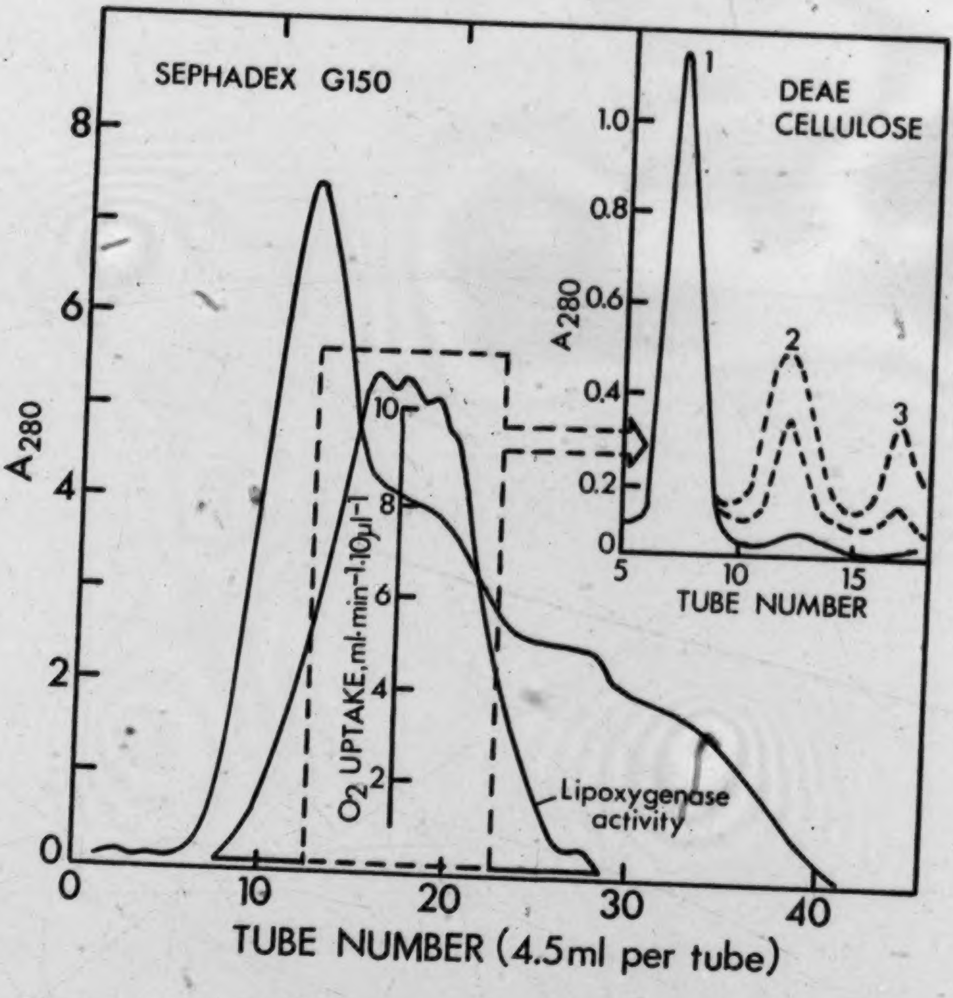


Figure 4. Purification of pea lipoxygenase according to Procedure I. Sephadex G 150 column effluent contained lipoxygenase activity concentrated in tubes 11-23, which were combined, precipitated with 50% ammonium sulfate saturation and dialyzed against Tris-Ca^{2+}-SH. Using the same buffer as eluent, one or two purifications were performed on a DEAE-cellulose column. The size of elution peaks 2 and 3 decreased substantially as the duration of the previous dialysis step was prolonged.

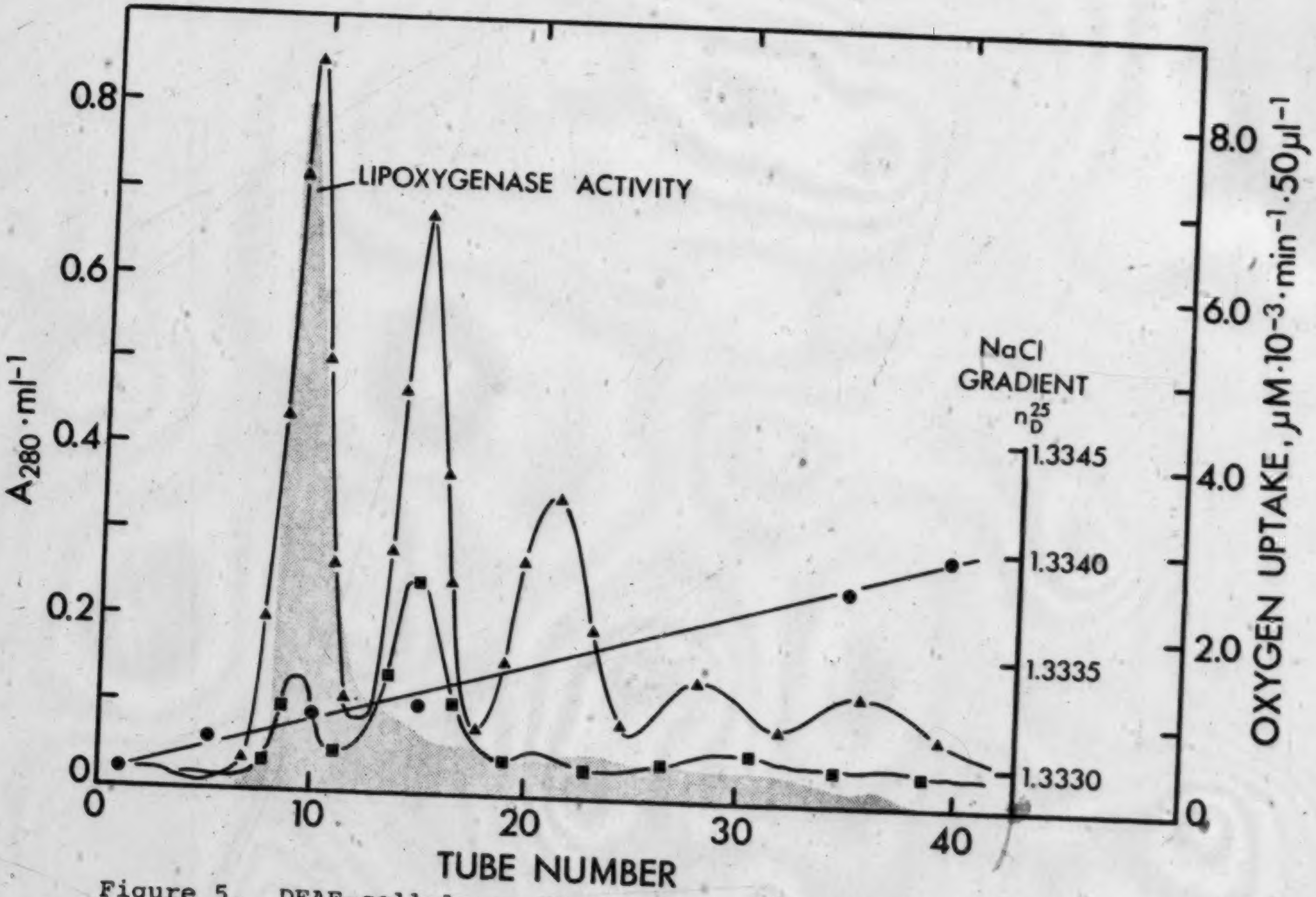


Figure 5. DEAE-cellulose column purification of pea lipoxygenase. Procedure I, -■-■-■-; Procedure II, -▲-▲-▲-.

by a factor of 3/2, to give 3.0 ml final volume, and wrapping in aluminum foil to exclude light.

Activity as a function of enzyme concentration.

Lipoxygenase (first DEAE-cellulose column effluent peak), prepared by Procedure I, was tested for activity by the oxygraph at 25°C. using the standard substrate and varying amounts of enzyme. Appropriate dilutions with 10 mM phosphate buffer, pH 7.0, were made of the enzyme preparation to allow injection of the same quantity (20 µl) into the cell, which was set up with 2.98 ml of substrate mixture. The undiluted preparation contained 46.2 A_{280} units $\cdot$ ml^{-1}. Thus, ten different concentrations, ranging from 0.924 A_{280} units down to 0.0014 A_{280} units per 3 ml of test solution, were assayed.

The effect of calcium

The paper published by Christopher et al. (1972), concerning isolation and characterization of a third isoenzyme from soybean lipoxygenase, suggested an approach to the study of pea seed lipoxygenase, particularly to the entity represented by the first elution peak from the DEAE-cellulose column. Conjecturing that this entity might represent one of the corresponding isoenzymes, oxygen uptake measurements were made on the enzyme preparation, using the standard substrate at 25°C. in the oxygraph with varying amounts of calcium ion. A stock solution of 0.1 M $CaCl_2 \cdot 2H_2O$ was employed to adjust the substrate mixtures to zero (control), 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM with respect to Ca^{2+}, respectively. The activity of the isoenzyme prepared in the presence of calcium (Procedure I) was compared with that of the isoenzyme prepared in its absence (Procedure II).

The effect of aging.

The stability of the enzyme prepared by Procedure III and stored in the refrigerator at 4°C. was tested periodically over 31 days (9 determinations), using the oxygraph at 25°C. with standard substrate.

The effect of cyanide

In order to establish the nature of the three peaks emerging from the DEAE-cellulose column (Procedure I), an investigation of the effect of 5 mM potassium cyanide on 50 µl-samples from each peak was undertaken. The oxygraph was used at 25°C. with the standard substrate to measure oxygen consumption, both with and without cyanide. The third peak was tested with 5 mM Ca^{2+}, also (see Figure 6). The same cyanide test was applied to the three major peaks of DEAE-cellulose column effluent, Procedure III (30 µl-samples).

The effect of iron and copper.

To investigate the effects of Cu^{2+}, Fe^{2+} and Fe^{3+} ions upon lipoxygenase (first peak from DEAE-cellulose column, Procedure I), stock solutions of $CuSO_4 \cdot 5H_2O$, $(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$ and $NH_4Fe(SO_4) \cdot 12H_2O$ were made up in

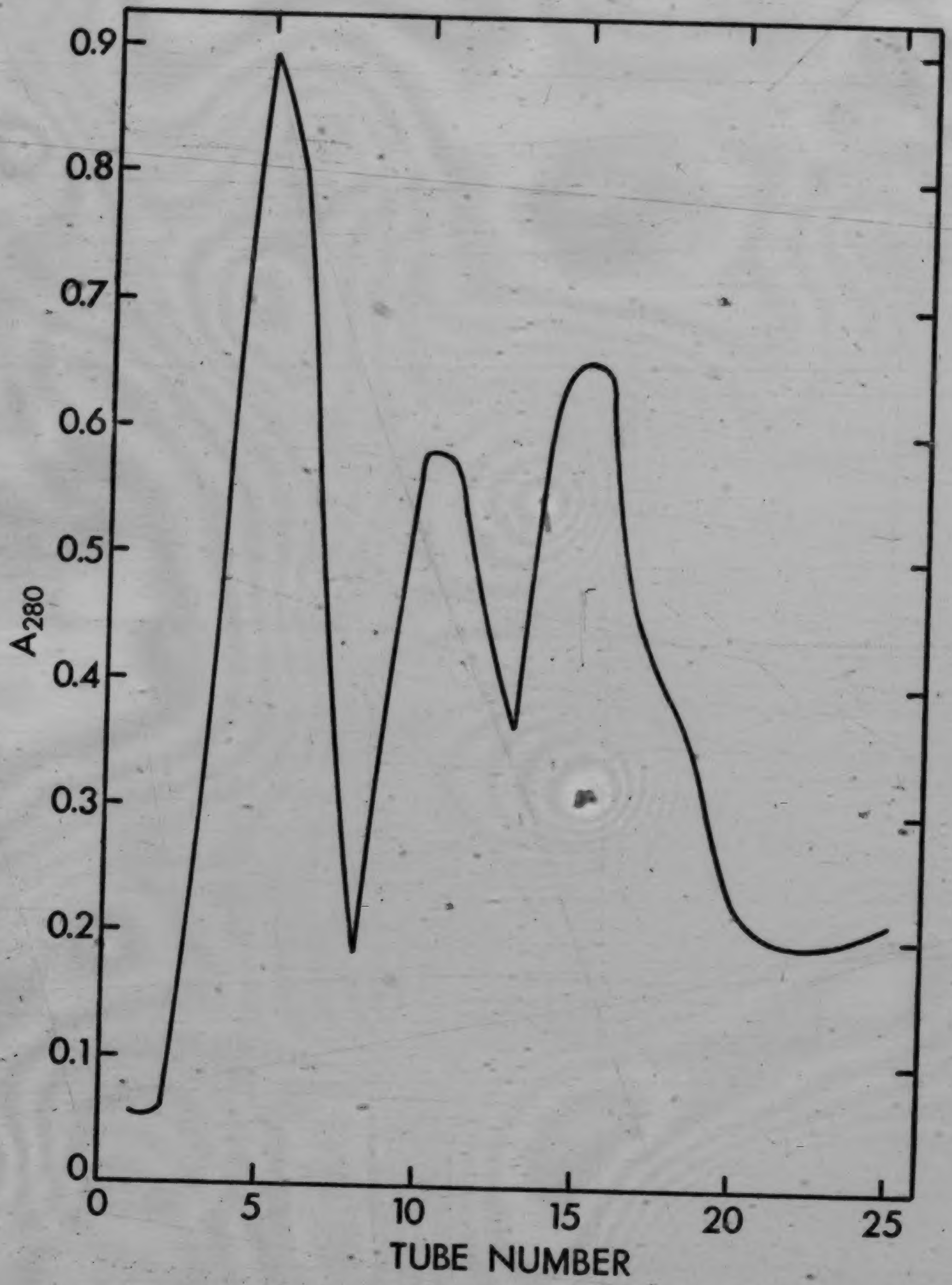


Figure 6. A_{280} of fractions of DEAE-cellulose column effluent according to Procedure I. Each tube contained 4.5 ml. Samples of 50 µl from tubes 5, 10 and 15 were tested for susceptibility to cyanide; tube 15 was tested for susceptibility to calcium.

distilled water to 2 mM (copper sulfate), 5 mM and 5 M (both iron ammonium sulfate salts). These metallic ion solutions, when added to the standard oxygraph substrate in the amount of 30 µl, were then diluted 100 to 1. Amounts of 50 µl of enzyme preparation containing 50 µg of protein were used in the assay (25°C.), which was designed to compare activity of substrate alone with that of substrate in the presence of each of the ions.

Substrate specificity and pH dependence.

The activity of lipoxygenase (first DEAE-cellulose column peak, Procedure II) as a function of pH was investigated for each of the three lipid substrates, linoleic acid, methyl linoleate and trilinolein. Final substrate concentrations of 20, 20 and 40 mg%, respectively, were achieved by adding appropriate amounts of stock lipid solutions to solutions of Tween-20 in buffer to yield a final volume of 3 ml for the oxygraph cell. Oxygen uptake measurements were made at 20°C. with 30 µl quantities of enzyme preparation (72 µg protein) per assay. Solvents used in stock solutions were ethanol (95%) for linoleic acid and acetone-ethanol (40%-60%, v/v) for the other two lipids. The ten different 50 mM buffer solutions containing 0.25% Tween-20 were prepared as follows: sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0, 6.5, 7.0, 7.5 and 8.0) and sodium borate (pH 8.5, 9.0 and 9.5).

Temperature coefficients.

To investigate the effect of temperature upon the activity of lipoxygenase (first DEAE-cellulose column peak, Procedure II), the standard oxygraph assay was applied at eight different temperatures, ranging from 5°C. to 40°C., with 5° intervals. A calibrated thermometer was used to establish each new temperature (±0.05°) with water in a second cell of the instrument, before the substrate mixture was added to the test cell. Then, ten minutes were allowed to elapse after introducing the substrate solution, before adding enzyme, to guarantee proper temperature equilibrium and solution of ambient air.

Heat stability.

The stability of lipoxygenase (first DEAE-cellulose column peak, Procedure III) was tested by holding samples for up to 20 min at 50°, 60°, 70°, 80° and 90°C. before determining activity in the oxygraph at 25°C., using the standard assay substrate and 13.1 µg of enzyme protein (as determined by the Lowry method). The active enzyme preparation, diluted 5 to 1 in 10 mM phosphate buffer, pH 7.0, was held at the desired temperature in a solid state heating block. The timer was started at the moment temperature was attained, aliquots were withdrawn sequentially at 1, 3, 5, 10, 15 and 20 min with a microsyringe and oxygraph measurements were undertaken right away. Duplicate samples of enzyme not exposed to heat were assayed also for the control.

b. Gel Electrophoresis.

Molecular weight determination.

Protein molecular weights were determined for pea lipoxygenase (Procedure I, first DEAE-cellulose column effluent peak), for commercial soybean lipoxygenase and for pea albumins and globulins (cf. Methods B.2.c. Albumins and Globulins) following essentially the procedures of Ornstein and Davis (1960) and Weber and Osborn (1969). Polyacrylamide gels (8.5%; Acrylamide-Bis ratio, 45:1) were formed in 8 x 100 mm glass tubes. The gels and electrode reservoirs contained 50 mM sodium phosphate buffer, pH 7.1, and 0.1% SDS. Protein-SDS complexes were prepared by heating sample solutions (containing approximately 4 to 7 μg of protein per anticipated separation band per gel) for ten minutes in a boiling water bath in the presence of 1% SDS and 0.5 mM dithiothreitol. After cooling, an equal quantity of 60% glycerol was added to increase the density of the protein solution (the stacking gel was omitted). Several samples of a mixture of six standard proteins were included in every run, both on separate gels and mixed with unknown proteins. The standards used were cytochrome c, chymotrypsinogen A, tropomyosin, ovalbumin, BSA and human γ-globulin (cf. Figure 17). Electrophoresis was performed at room temperature using a current of 5 mA per tube for 5 h. The gels were stained for 2 h in 0.2% (w/v) Coomassie Brilliant Blue dissolved in 9.2% aqueous acetic acid containing 46% methanol. Destaining was performed electrophoretically in 7.5% aqueous acetic acid containing 5% methanol over a period of 20 min, with a change of destaining solution after 10 min (cf. Evaluation of Calibration Curve Used in Determination of Molecular Weights, Appendix).

Zymograms

Isoenzyme patterns of lipoxygenase, isolated from fresh green peas and pea seeds by Procedure I, were obtained on 7% polyacrylamide gels (Acrylamide-Bis ratio, 38:1) with the technique reported by Guss *et al*. (1967). This is a modification of the Ornstein-Davis technique, which employs 1% potato amylose in the small pore gel. Both stacking gel and 30% glycerol were used in the application of 100 μg to 200 μg of protein per tube, without any evident difference in observed bands. Electrophoresis was carried out in the cold room, for approximately 3 h, until tracker dye (5 mg% Bromphenol Blue) was within 1 cm of the end of the gel. Reservoirs were filled with aqueous Tris-glycine buffer (0.025 *M* - 0.19 *M*), pH of 8.3. Current was maintained at 4 mA per tube. The staining of the gels containing active enzyme entities was accomplished after 7.5 *mM* linoleic acid in 0.1 *M* Tris-Cl, pH 8.3, containing 0.25% Tween-20 (freshly sonicated at a power level setting of 8, tuned to 6 A.) was reacted with the gels for 30 min. Parallel substrate solution containing 5 mM KCN were also used. Following exposure of enzyme to substrate, staining with saturated aqueous KI solution was accomplished in 5 to 20 min.

The enzyme extracts in buffer solutions of pH 7.0 or pH 6.5 were applied without preliminary readjustment of their pH, nor was preliminary or simultaneous treatment of gels with thioglycollic acid performed (Hale *et al.*, 1969). To avoid the possibility of obtaining an altered electrophoretic pattern due to an interaction of pea lipoxygenase with free fatty acids, defatted enzyme samples were also run. However, when the crude enzyme was extracted with cold $CHCl_3$-CH_3OH (2:1, v/v), followed by acetone, the isoenzyme pattern was similar to that of unextracted enzyme (Haydar *et al.*, 1975). As this observation is in agreement with results obtained by Hale et al. (1969), the defatting step was omitted and the zymograms reported are for *in situ* isoenzymes.

3. Carotene Destruction Mediated by Lipoxygenase

The technique of Ben Aziz *et al.* (1971) for following the rate of destruction of β-carotene in an aqueous medium containing linoleic acid, lipoxygenase and Tween-20 was used. The detailed instructions were followed for reaction mixtures containing 1.4×10^{-5} M β-carotene and certain modifications were introduced to afford final concentrations, both greater and smaller, as follows:

	β-Carotene Concentration			Concentration Linoleic Acid
Reaction Mixture*	$\mu g \cdot ml^{-1}$	M	Relative to c	M
a	1.875	3.4×10^{-6}	0.25 x	2.0×10^{-3}
b	3.75	6.8×10^{-6}	0.5 x	2.0×10^{-3}
c	7.5	1.4×10^{-5}	1 x	2.0×10^{-3}
d	15	2.7×10^{-5}	2 x	2.0×10^{-3}
e	22.5	4.1×10^{-5}	3 x	2.0×10^{-3}

* Concentrations are based on a final volume of 3 ml.

Details for solution preparation are as follows:

Aqueous Linoleate

Ethanolic linoleic acid (1.0 ml; 7.5%, w/v) was mixed with 0.3 ml of ethanolic Tween-80 (10%, v/v). Aqueous EDTA·Na_2 (5.0 ml; 0.5%, w/v) was added. The pH was adjusted to 9.0 by dropwise addition of 0.1 N NaOH and the volume adjusted to 10 ml with distilled water. This solution was stable for one week under N_2 in the refrigerator (4°C.).

Aqueous Carotene

β-Carotene, 25 mg, plus 0.9 ml Tween-80 were dissolved in 25 ml $CHCl_3$ (when not used immediately, the solution was stored under N_2 in the refrigerator, protected from light, up to 48 h). Of this solution, 1.0 ml was evaporated to dryness under a stream of N_2, in diminished light, and the residue was dissolved immediately in 10 ml of aqueous EDTA·Na_2 (0.25% w/v). This solution was prepared fresh daily.

Buffer Solution

Citrate-phosphate buffer, 0.2 M, pH 7.0.

Aqueous Buffered Carotene-Linoleate Solution

Aqueous linoleate, aqueous carotene and buffer solutions were combined in volume ratios 1:1:8, giving reaction mixture c (Ben Aziz *et al.*, 1971). Since an increase in the proportion of β-carotene in the aqueous carotene solution was impossible (solubility level would be surpassed), the increase in final concentration was effected by increasing the proportions of aqueous carotene, while holding the final linoleic acid concentration constant. Hence, reaction mixtures a, b, d and e were modifications of the original (c):

Reaction Mixture	Aqueous Linoleate	Aqueous β-Carotene	Buffer Solution
a	4 ml	1 ml	35 ml
b	2 ml	1 ml	17 ml
d	1 ml	2 ml	7 ml
e	1 ml	3 ml	6 ml

Since parallel determinations were to be done on reaction mixtures in 1-cm spectrophotometer cells (total capacity

4 ml) and in an oxygraph cell containing 3 ml of reaction medium, 3 ml was selected as total volume in each case. Therefore, 2.25 ml of aqueous buffered carotene-linoleate solution were placed in the cell and 0.75 ml enzyme solution (pea lipoxygenase represented by the first peak from the DEAE-cellulose column, Procedure I) was added at zero time for measuring carotene destruction. With the oxygraph cell, however, only a small quantity of enzyme solution can be added at zero time. Thus, 2.25 ml of aqueous buffered carotene-linoleate plus 0.72 ml of water were added three minutes before addition of 32.6 μl of enzyme solution to allow temperature and gaseous equilibrium to be reached. The oxygraph is equipped with a magnetic stirrer, but the only mixing possible in the spectrophotometer cell is provided by the vigorous squirt from the syringe of the enzyme solution at zero time. In both techniques, syringes were withdrawn as quickly as possible to permit the manipulations required for excluding light to follow immediately. Temperatures were strictly controlled (25.00±0.05°C.).

To provide some data for comparison, commercial soybean lipoxygenase was also used.

A preliminary scan of the aqueous buffered carotene-linoleate solution, 3 parts, plus 1 part water (no enzyme) was carried out in the 420 to 500 nm region in order to verify the λ_{max} at 460 nm (See Figure A3). Another preliminary study with the isolated pea and commercial soybean lipoxygenase was carried out to test bleaching efficiency at two different enzyme levels, 10 μg·ml^{-1} and 250 μg·ml^{-1}. The former is the concentration used in the published method which was being rehearsed and the latter resulted from use of the undiluted enzyme preparation available from this study (920 μg·ml^{-1} diluted 4 times in the cell).

On the basis of these findings, the study of carotene destruction was undertaken over 15 min periods in the recording spectrophotometer at λ = 460 nm, employing the five different concentrations described. The 10 μg ·ml^{-1} level of each enzyme was adhered to. The enzyme solutions were added at zero time after balancing the instrument with substrate-plus-water solution made up with all constituents for aqueous carotene solution except β-carotene. The rates of decrease in absorbance were computed from the strip charts. These data, in turn, were converted into μg β-carotene loss · min^{-1}. Finally, μg β-carotene · mg enzyme^{-1} was computed for each enzyme on a 15-second incremental basis and on a cumulative basis.

Oxygraph determinations were made upon duplicate reaction mixtures. Only initial rates of oxygen uptake were obtained by this method. In all carotene destruction studies, light was excluded or diminished as much as possible.

B. Model Systems for the Study of Non-Enzymic Oxidations.

1. Preliminary Experiments

a. Filter paper, linoleic acid and the carotenoids, β-carotene and crocin.

All substrates except crocin were described under *Materials and Chemicals*. The method of Friend and Mayer (1960) was employed to extract crocin from saffron. Briefly, it entailed the removal of lipid and other carotenoids by continuous ether extraction at room temperature in the dark, removal of ether, and extraction of the residue with methanol. The methanol solution was kept in the refrigerator as a stock solution. Crocin was freed from methanol by evaporating an aliquot of the solution to dryness while protecting from light and from excessive heat. The residue was redissolved in water to a known volume. From the known molecular weight, 977, the volume of the methanolic aliquot, and $A^{1\%}_{1cm} = 1369$ at 440 nm (Isler *et al.*, 1957), the concentration of aqueous crocin and, hence, that of the stock solution, was determined. The absorption spectrum (in water) was obtained at the same time to verify the purity. The maxima at 440 nm and 473 nm are consistent with the published data (see Figure A4).

Filter paper discs (3.0 cm diameter) were impregnated in methanolic solutions containing linoleic acid

(10% w/v; each disc absorbed 3.6 x 10^{-4} mole of linoleic acid on the average) and crocin at the desired molarity. Comparable matrices, impregnated with linoleic acid and β-carotene, were prepared from $CHCl_3$ solution. Molarities employed for carotenoids were zero (controls), 10^{-8}, 10^{-6} and 10^{-4}.

Chloroform was purified before use by copious washing with water in a separatory funnel (4 times with distilled water and 2 times with de-ionized distilled water) in order to remove phosgene and HCl, vigorous shaking with $CaCl_2$, to remove H_2O, traces of ethanol and traces of phosgene, and subsequent distillation over $CaCl_2$ into a brown bottle for storage in the cold room.

Once the filter paper matrix discs were impregnated with the appropriate solutions by immersion, individually with forceps, for 10 seconds, followed by draining on the inside wall of the beaker, they were air-dried for 10 min on stainless steel wire-mesh racks, freeze-dried for 24 h at a pressure <5μ, and stored in desiccators in the cold, protected from light.

Gilson respirometer readings were taken at 25°C. over 5 h-periods. Each 15 ml flask received four carefully crumpled discs (i.e., not torn, nor folded with creasing). All manipulations of discs were done with forceps. The work was carried out rapidly in diminished light and flasks were sealed to manometer glass joints with high vacuum silicone grease and modelling clay. Finally, they were covered with aluminum foil to exclude light. Periodic flushing of the system with dry air (twice in 5 h) in order to guarantee excess of oxygen was performed by means of a water tap-aspirator, air being admitted through a U-tube packed with dry $CaCl_2$. Four or five flasks were used for each matrix category and the values were averaged (cf. Note Concerning Assessment of Precision of the Gilson Respirometer, Appendix). Manometer adjustments were made every 30 to 60 min and readings recorded approximately every 2 h. All glassware, wire racks and forceps were scrupulously cleaned with alcoholic KOH or detergent, followed by thorough rinsing, ending with distilled water, and oven dried.

For comparison, determinations of oxygen uptake from solution by means of the oxygraph were made on enzymically catalyzed oxidations of linoleic acid in the presence of crocin and β-carotene. The substrate (2.90 ml) contained 7.5×10^{-3} $\underline{M}$ linoleic acid, 0.25% Tween-20 (v/v), $\underline{M}$/75 phosphate buffer, pH 7.0 and the enzyme preparation (0.10 ml) contained $0.125 \text{ mg} \cdot \text{ml}^{-1}$ of commercial soybean lipoxygenase in 0.1 $\underline{M}$ phosphate buffer, pH 7.0.

b. Wheat proteins, linoleic acid and β-carotene.

Wheat protein fractions were dissolved in their appropriate solvents (see under Materials and Chemicals) to the extent of 1% (w/v) for filter paper disc impregnation. After freeze-drying, as described under a, they were impregnated with 10% (w/v) linoleic acid in $CHCl_3$ or 10% (w/v) linoleic acid plus 10^{-4} $\underline{M}$ β-carotene in $CHCl_3$, again subjected to freeze-drying and stored in desiccators in the cold and in the dark. Respirometer studies, with the exclusion of light, were carried out at three different temperatures (25°, 40° and 50°C.) over 50 h. One disc was used per flask and results were tabulated as cumulative through time. Zero settings were chosen at 100 or 200 µl rather than 0 µl, because many matrices exhibited net evolution, i.e., increase of gas phase volume at constant pressure, early in the run. Air flushing was performed every 5 h in the beginning of a run, becoming more frequent (2 or 3 h intervals) toward the end, when oxidation increased.

2. Oxidation of Pea Lipids on Some Pea Constituents as Matrices.

a. Measurement of oxidation.

Oxidation was monitored by means of Gilson differential respirometers in the dark (aluminum foil applied to each flask) and in intense light, at 50°C.

Although no means were at hand to allow precise definition of light intensity, all experiments in light were run under identical illumination. The eight 30-

watt reflector flood lamps of the Gilson GP 8 were combined with the overhead lighting of the room and a supplementary UV lamp, suspended over the water bath. The room was illuminated by two fluorescent lamp units, the first being directly centered over the respirometer; the second, parallel with it and at the same height from the floor (2.43 m), was 1.57 m distant from the first. Each unit bore two 40-watt (cool) tubes, 1.18 m long, all four tubes parallel to each other and the two rows of flasks. The bottoms of the flasks containing matrix discs were, thus, 1.39 m and 2.10 m removed from the first and second overhead units, respectively, all receiving the same intensity by the arrangement. In addition, the rectangular parabolic reflector of the UV lamp was placed in a centered position in line with the two rows of flasks, so that the rays fell approximately equally on all flasks. The height of the UV lamp of 20 cm above the water bath was found to provide optimal illumination to the flasks while shielding overhead light from the flasks uniformly. Illumination was continuous throughout the 50 h runs.

The basic technique employed was the same as for the preliminary experiments, including periodic air flushing and cumulative data tabulation. Four discs were used per flask.

From previous experience in this laboratory (Haydar and Hadziyev, 1973b, p.777), it was known that pea globulins combined with pea polar lipids exhibited the highest O_2 uptake at 50°C. in the light. Assuming the same to occur in the dark, head space analysis of oxygen with the Beckman Laboratory Oxygen Analyzer, Model 777, was carried out on air surrounding matrices of pea polar lipids + β-carotene + pea globulins in the dark for 50 h at 50°C. Also, 50 h-runs of polar lipids + β-carotene impregnated on globulins, albumins, amylose, amylopectin, pectin and cellulose respectively, were carried out in the intensely lighted Gilson respirometer constant temperature water bath.

All head space oxygen determinations were done as follows. Three matrix discs, carefully crumpled, were placed in the 50 ml glass flask. The flask was immersed, unsealed, for 10 min in the 50°C. bath to allow complete temperature equilibrium to be attained. At the commencement of the 50 h Gilson run, the oxygen probe was sealed into place with high vacuum silicone grease and the barometer pressure was noted. The seal remained intact throughout the run. Although matrices were subjected to the same temperature and illumination (or darkness) as those in the Gilson flasks, there was the fundamental difference that the head space analysis flask and oxygen probe could not be shaken, and therefore remained stationary. Pure N_2 was used to check the efficiency of the oxygen probe, a reading of 1% to 3% O_2 saturation (normal ambient air as 100%) being accepted as an adequate zero point, since oxygen contamination could not be avoided completely.

b. Lipids

Homesteader pea seeds, 1000 g, were ground as above and soaked overnight in a 3-liter round bottom flask in about 2 l of $CHCl_3$:CH_3OH (2:1, v/v). The mixture was refluxed for 15 min, cooled and filtered. The solid mass was extracted twice again, with refluxing and filtration, until free of pigment. After combining the extracts, the volume was reduced to 500 ml by vacuum flash rotary evaporation (precautions were taken to avoid temperatures above 30°C. and exposure to light in this and all subsequent manipulations). Water was removed by addition of 99% pure ethanol and evaporation of the azeotropic mixture (100 ml first, followed by two additions of 50 ml each). After flash evaporation to dryness, the residue was dissolved in 100 ml of pure CH_3OH, mixed thoroughly at 90°C. and evaporated to dryness in a stream of N_2. This procedure, designed to break any existing lipid-protein bonds, was repeated twice more, using 50 ml of CH_3OH. The residue, crude total lipids, was dissoved in anhydrous $CHCl_3$ and stored in the dark at 4°C.

For purification of total lipids, the procedure of Folch et al. (1957) was followed. For subsequent separation of lipids into polar (PL) and neutral lipid (NL) fractions, column chromatography was applied, using silicic acid as adsorbent. Salicic acid and Hyflo Super Cel were washed, separately, in hot methanol, followed by hot acetone, and dried at 105°C. for 2 h. A 2.2 x 40 cm column was packed with a chloroform slurry of three parts, by weight, of silicic acid and one part Hyflo Super Cel. The total lipids, in $CHCl_3$, were applied to the column in the ratio of 30 mg/g silicic acid. NL and carotenoids were eluted together with 500 ml of pure $CHCl_3$. This solvent was removed by flash evaporation and the residue was dissolved in pet.ether. PL were eluted with 700 ml of $CHCl_3$-CH_3OH (1:1, v/v). No further fractionation was performed on the PL and they were freed of solvent, weighed, made up to 10% solution in $CHCl_3$ and stored in the cold (Cf. Figure 7).

Further chromatography on an alumina column was required to separate NL from carotenoids. A large column, 2.0 x 20 inches, was packed with a pet.ether slurry of Al_2O_3, which had previously been washed with pet.ether. The void volume, including sea sand, was 500 ml. The sample was applied in pet.ether and eluted with pet.ether containing at first, 1% acetone (v/v), with increasing amounts, up to 6% acetone. It was established that 2.5% acetone in pet.ether was the most efficient eluent. Thus, after the emergence of 800 ml of effluent, the column was

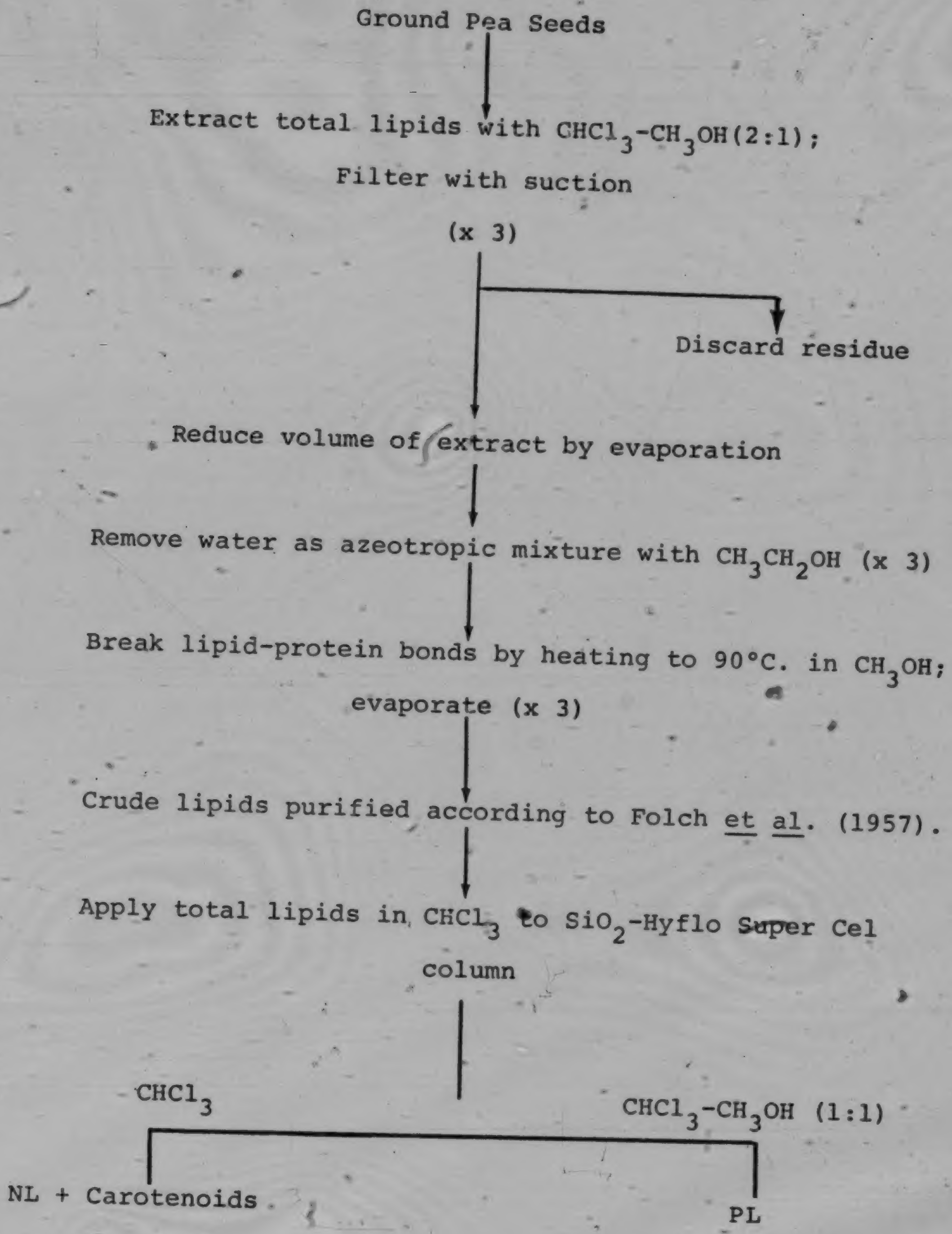

Ground Pea Seeds
Extract total lipids with $CHCl_3$-CH_3OH(2:1);
Filter with suction
(x 3)
Discard residue
Reduce volume of extract by evaporation
Remove water as azeotropic mixture with CH_3CH_2OH (x 3)
Break lipid-protein bonds by heating to 90°C. in CH_3OH;
evaporate (x 3)
Crude lipids purified according to Folch et al. (1957).
Apply total lipids in $CHCl_3$ to SiO_2-Hyflo Super Cel
column
$CHCl_3$
$CHCl_3$-CH_3OH (1:1)
NL + Carotenoids
PL

Figure 7. Flow diagram for isolation of NL + carotenoids and PL from peas.

just cleared of visible carotenes, the xanthophylls remaining at the top of the column. The next 1000 ml of effluent were assumed to contain NL. Final elution was performed with 350 ml of 6% acetone in pet.ether, the highest percentage which did not cause xanthophylls to move and which was assumed to elute some NL, as well. Since, after reduction of volume by flash evaporation, a residual yellowish coloration was evident in the combined NL fractions, a final purification was done with activated carbon.

The concentrated NL fraction was evaporated to dryness in a N_2 stream and redissolved in 100 ml of pure $CHCl_3$ in a 250 ml Erlenmeyer flask. Activated carbon was added in the amount of 0.2% by weight. The flask was stoppered with a cork and shaken continuously for 10 min. Then, suction filtration was performed, followed by washing the filter paper with fresh $CHCl_3$. This procedure was repeated, because no apparent discoloration was occurring. After three washings, A_{450} was read and found to be 0.435. After three more washings, in which the proportion of activated carbon was increased to 0.5% and finally to 1.0%, the A_{450} was 0.060, and the solution was nearly colorless. Ascribing the faint orange-red coloration to NL themselves, the treatment was stopped, since further treatment would risk depletion of NL and/or their oxidation. The solvent was removed, the NL weighed, made up to 10% solution in fresh $CHCl_3$ and stored at 4°C. protected from light. (cf. Figure 8).

c. Albumins and globulins

Total pea proteins were extracted and isolated as two fractions, albumins and globulins, according to Danielsson (1949, 1950) and Danielsson and Lis (1952).

Homesteader pea seeds (100 g) were ground to a fine powder with dry ice in a Waring blendor and extracted overnight by stirring with 350 ml of 0.05 M phosphate buffer, pH 7.0, containing 0.2 M NaCl. The slurry was filtered through cheesecloth and the homogenate was centrifuged at 10,000 x g for 10 min. Proteins in the supernatent were precipated overnight with 70% ammonium sulfate saturation and centrifuged at 10,000 x g for 10 min. The pellet was dissolved in 40 ml of the extraction buffer and dialyzed vs. running tap water overnight, followed by distilled water for 5 h, and finally vs. de-ionized distilled water for 15 h to remove traces of chloride. The dialyzate, containing protein, was centrifuged at 10,000 x g for 10 min to yield the globulins pellet (vicilin and legumin) and the albumins, held in the supernatent. The latter was dialyzed for an additional 5 h vs. de-ionized water and centrifuged at 10,000 x g for 10 min. The supernatent contained pure albumins. The pellet, containing globulins, was combined with the previous pellet and dialyzed vs. de-ionized water for 5 h. The dialyzate was

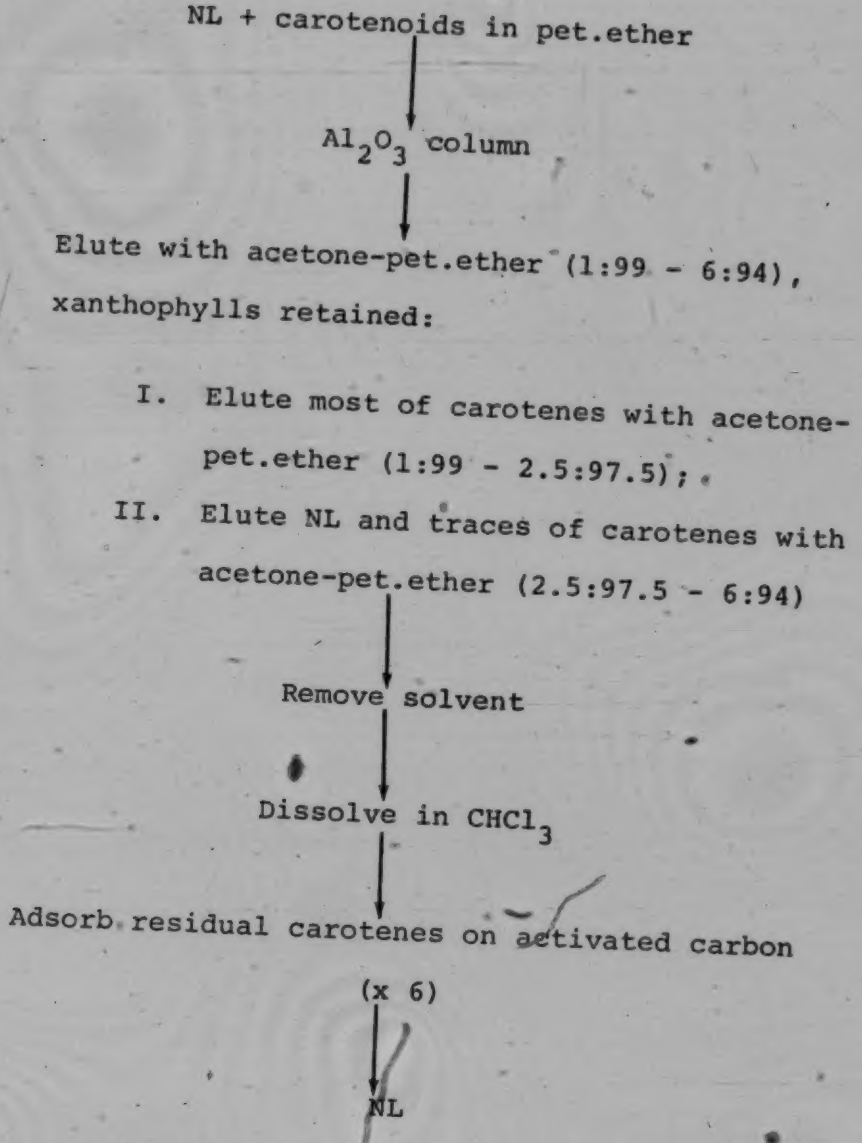

NL + carotenoids in pet.ether
Al2O3 column
Elute with acetone-pet.ether (1:99 - 6:94), xanthophylls retained:
I. Elute most of carotenes with acetone-pet.ether (1:99 - 2.5:97.5);
II. Elute NL and traces of carotenes with acetone-pet.ether (2.5:97.5 - 6:94)
Remove solvent
Dissolve in CHCl3
Adsorb residual carotenes on activated carbon
(x 6)
NL

Figure 8. Flow diagram for separation of NL from carotenoids.

centrifuged at 10,000 x g for 10 min and the pellet, consisting of pure globulins, was dissolved in a few ml of 0.2 M NaCl. All of the fore-going procedures were carried out in the cold (4°C.).

The two fractions were freeze-dried for 24 h at a pressure <5μ. The protein isolation steps are outlined in Figure 9. Gel electrophoresis of both protein fractions was performed as previously described for lipoxygenase.

d. Carbohydrates

Besides the cellulose matrix represented by filter paper itself, three other carbohydrates were employed as matrices by impregnation of filter paper discs in 1% (w/v) aqueous solutions of pure amylose, amylopectin and slow setting pectin.

e. Matrix preparation

Upon the six basic matrices, albumins, globulins, cellulose, amylose, amylopectin and pectin, were superimposed Nl, NL + β-carotene, PL and PL + β-carotene by repeat impregnation and freeze-drying. The latter impregnations were performed in $CHCl_3$ solutions at 4°C.

Lynch et al. (1959) reported a range of 0.4 to 0.6 mg of β-carotene per 100 g fresh peas having a moisture content of 77.5%. From the mean of this range (0.5 mg · 100 g fresh peas^{-1}), the NL/PL ratio of 1.8 and total

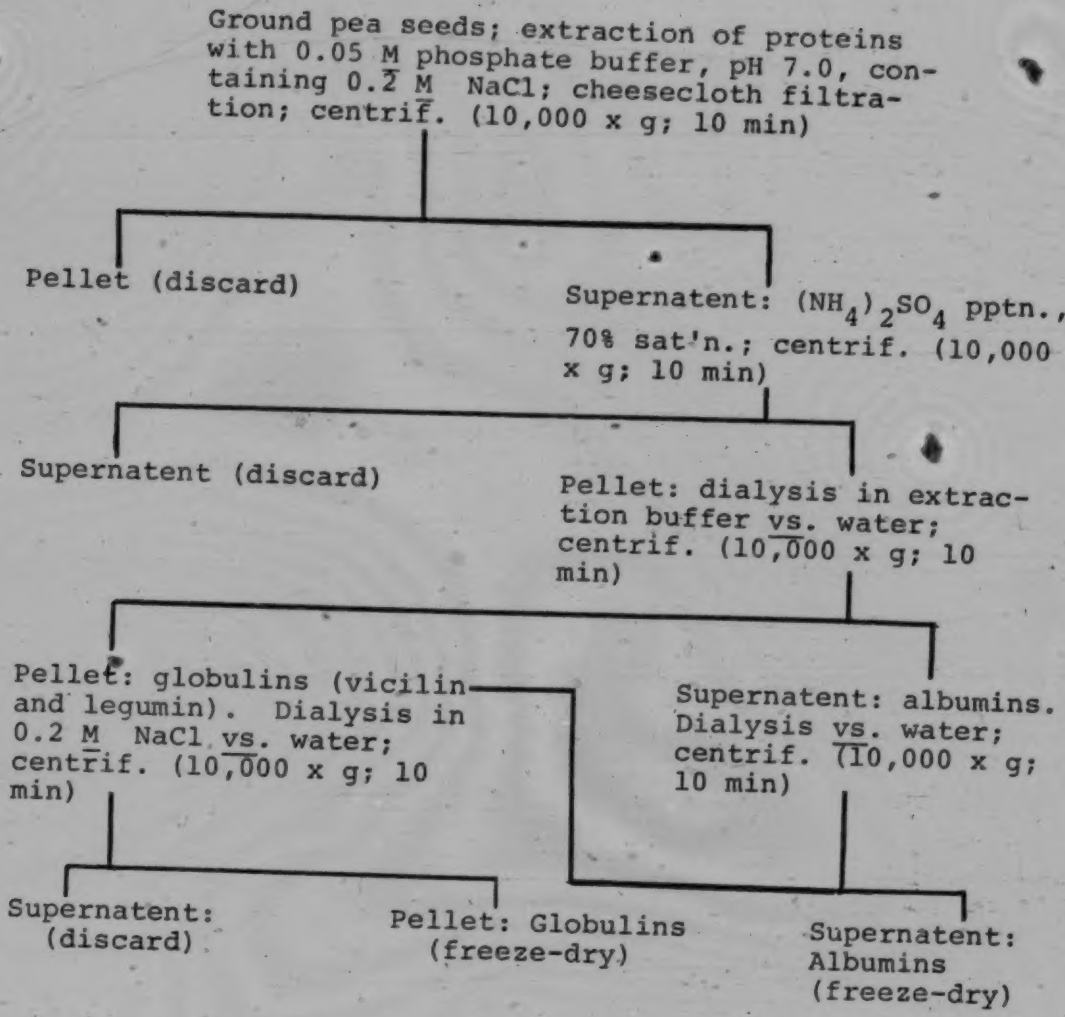

Ground pea seeds; extraction of proteins with 0.05 M phosphate buffer, pH 7.0, containing 0.2 M NaCl; cheesecloth filtration; centrif. (10,000 x g; 10 min)
Pellet (discard)
Supernatent: $(NH_4)_2SO_4$ pptn., 70% sat'n.; centrif. (10,000 x g; 10 min)
Supernatent (discard)
Pellet: dialysis in extraction buffer vs. water; centrif. (10,000 x g; 10 min)
Pellet: globulins (vicilin and legumin). Dialysis in 0.2 M NaCl vs. water; centrif. (10,000 x g; 10 min)
Supernatent: albumins. Dialysis vs. water; centrif. (10,000 x g; 10 min)
Supernatent: (discard)
Pellet: Globulins (freeze-dry)
Supernatent: Albumins (freeze-dry)

Figure 9. Flow diagram for isolation of pea albumins and globulins.

lipid content of 3%, dry basis, for Homesteader pea seeds (Haydar, 1972) and the moisture content of Homesteader pea seeds of 15.93% (see Materials and Chemicals), the following ratios of β-carotene to lipid fraction were calculated:

$$0.0010 \text{ g } \beta\text{-carotene} \cdot \text{g NL}^{-1} \quad \text{and}$$

$$0.0018 \text{ g } \beta\text{-carotene} \cdot \text{g PL}^{-1}.$$

These ratios were applied in matrix disc impregnations by adding the appropriate amounts of β-carotene to 10% (w/v) $CHCl_3$ solutions of NL and PL.

IV. RESULTS

A. Lipoxygenase Activity.

1. Characterization

Pea lipoxygenase activity contained in the first A_{280} peak of DEAE-cellulose column effluent varied considerably according to method of preparation, as Table 2 shows. In Procedure I, in which the ion exchange column was eluted with Tris-Ca^{2+}-SH buffer, the resulting enzyme preparation caused uptake by oxygraph substrate of 4.59 $\mu\underline{M}$ $O_2 \cdot \text{min}^{-1} \cdot \text{mg enzyme}^{-1}$. This activity was about 1/8 (12.8%) of that of the corresponding preparation from Procedure II, in which calcium and Sephadex filtration were omitted, and about 1/3 (34.5%) of that of the corresponding preparation from Procedure III, in which ammonium sulfate precipitation was omitted and both chromato-

Table 2. Pea Lipoxygenase Activities Expressed as Rate of O_2 Uptake Per Milligram Protein (Lowry Method) in the Standard Oxygraph Assay*

Procedure	$\mu\underline{M}\ O_2 \cdot min^{-1} \cdot mg^{-1}$
I	4.59
II	36.0
III	13.3

* Conditions:

Substrate: 7.5×10^{-3} $\underline{M}$ linoleic acid in 0.1 $\underline{M}$ Tris-Cl buffer, pH 7.0, containing 0.25% Tween-20 (v/v).

Enzyme Preparation: First DEAE-cellulose column effluent peak.

Temperature: 25°C.

graphic steps were retained. The Procedure III preparation reported in Table 2 was purified once only on DEAE-cellulose. Thus, Procedure II appears to yield the preparation with highest activity.

Investigation of enzyme activity as a function of enzyme concentration (Procedure I, first DEAE-cellulose column effluent peak) yielded the data for Figure 10. In Figure 10, activity is seen to depart from linearity over the broad concentration range tested (up to 9.24 x 10^{-1} A_{280} unit per assay). Of the ten different concentrations assayed, only the first four (lower range) conformed to a straight line relationship. Protein content of the enzyme preparation (Lowry method) gave a calcualted value of 1.07 mg protein per ml per A_{280} unit.

In Figure 11, the activity of lipoxygenase (first DEAE-cellulose column effluent peak) prepared in the presence of calcium is seen to be considerably less than that of lipoxygenase prepared in its absence (the first two values shown in Table 2 correspond to the zero levels of Ca^{2+} added to substrate for enzyme preparations $+Ca^{2+}$ and $-Ca^{2+}$, respectively). Figure 11 reveals further that increasing amounts of calcium added to the substrate exhibit increasing inhibition, regardless of previous treatment. Indeed, in the lower activity regions, the curves appear to be equivalent, but out of phase by 2.5 mM Ca^{2+}. In other words, the addition of 2.5 mM Ca^{2+} to substrate,

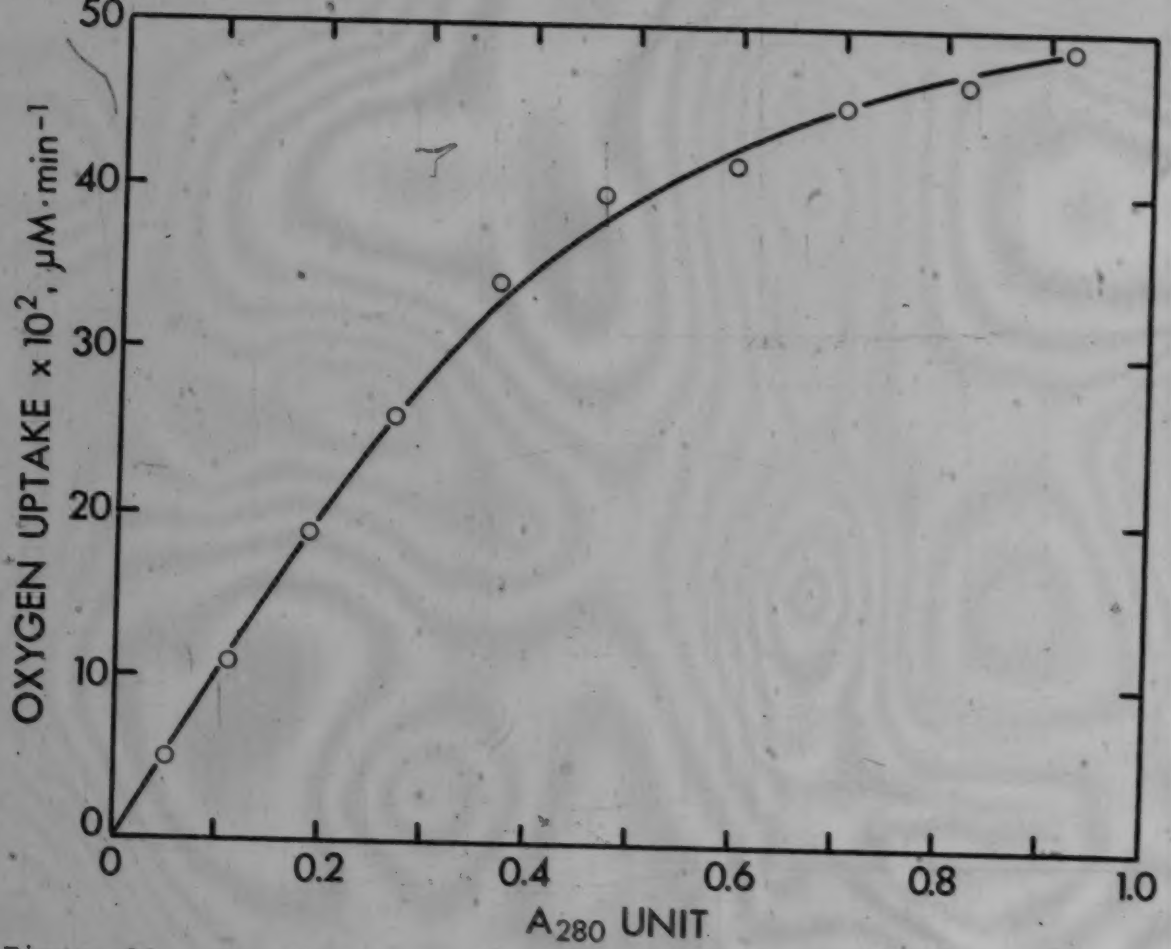


Figure 10. Pea lipoxygenase activity as a function of enzyme concentration. Standard oxygraph assay of oxygen uptake at 25°C. was performed on enzyme preparations contained in the first DEAE-cellulose column effluent peak, Procedure I.

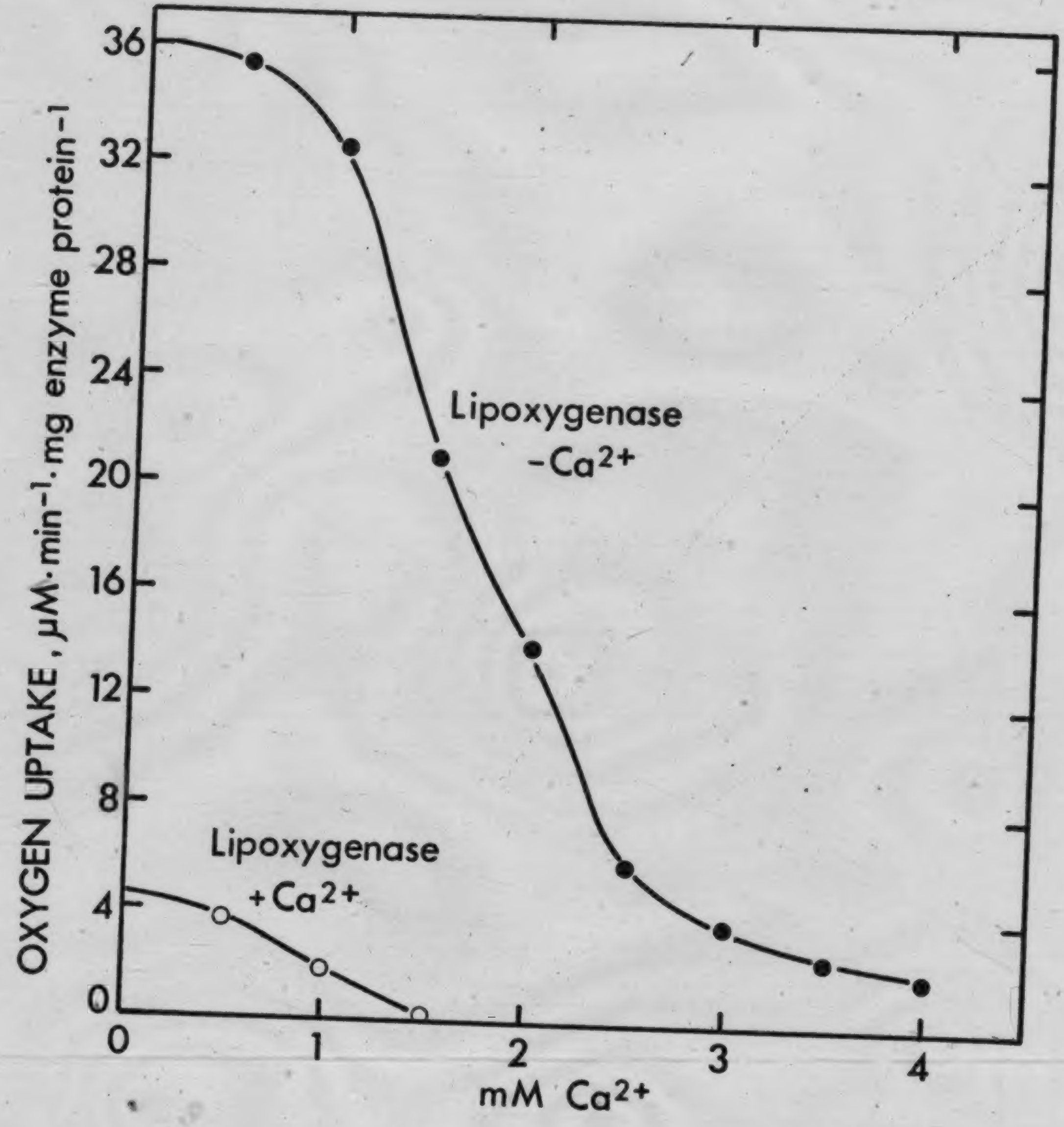


Figure 11. Comparison of the effects of increasing amounts of calcium upon pea lipoxygenase activity contained in the first DEAE-cellulose column effluent peak, prepared in the absence of calcium ($-Ca^{2+}$) and in its presence ($+Ca^{2+}$). Standard oxygraph assays performed at 25°C.

seems to render the $-Ca^{2+}$ enzyme preparation as inhibited as the other $(+Ca^{2+})$.

Figure 12 indicates that activity as a function of aging in cold storage decreases by 29.1% over 31 days, more than one third of this decrease (36%) occurring in the first 24 hr.

The effects of 5 mM cyanide on the activity of the enzyme components prepared according to Procedures I and III are summarized in Table 3. A high degree of inhibition of the third peak by 5 mM Ca^{2+} is also indicated (Procedure I). Figure 13 shows the concentration of enzyme activity to be in the second major peak of DEAE-cellulose column effluent when Procedure III is employed. This activity was inhibited 80% by 5 mM cyanide; lipoxygenase activity in the first and third major peaks was inhibited 80% and 100%, respectively, by 5 mM cyanide (Table 3).

Table 4 shows the effects of iron and copper ions upon the activity of lipoxygenase isolated by Procedure I (first DEAE-cellulose column effluent peak). It is noted that the higher concentrations of the iron salts caused significant catalysis without the addition of enzyme and that the relatively low copper concentration (20 µM Cu^{2+}) was very inhibitory (>95%). Both Fe^{2+} and Fe^{3+} stimulated enzyme activity at the level of 50 µM in the substrate, but they each inhibited when present at levels of 50 mM.

Substrate specificity and pH dependence for the

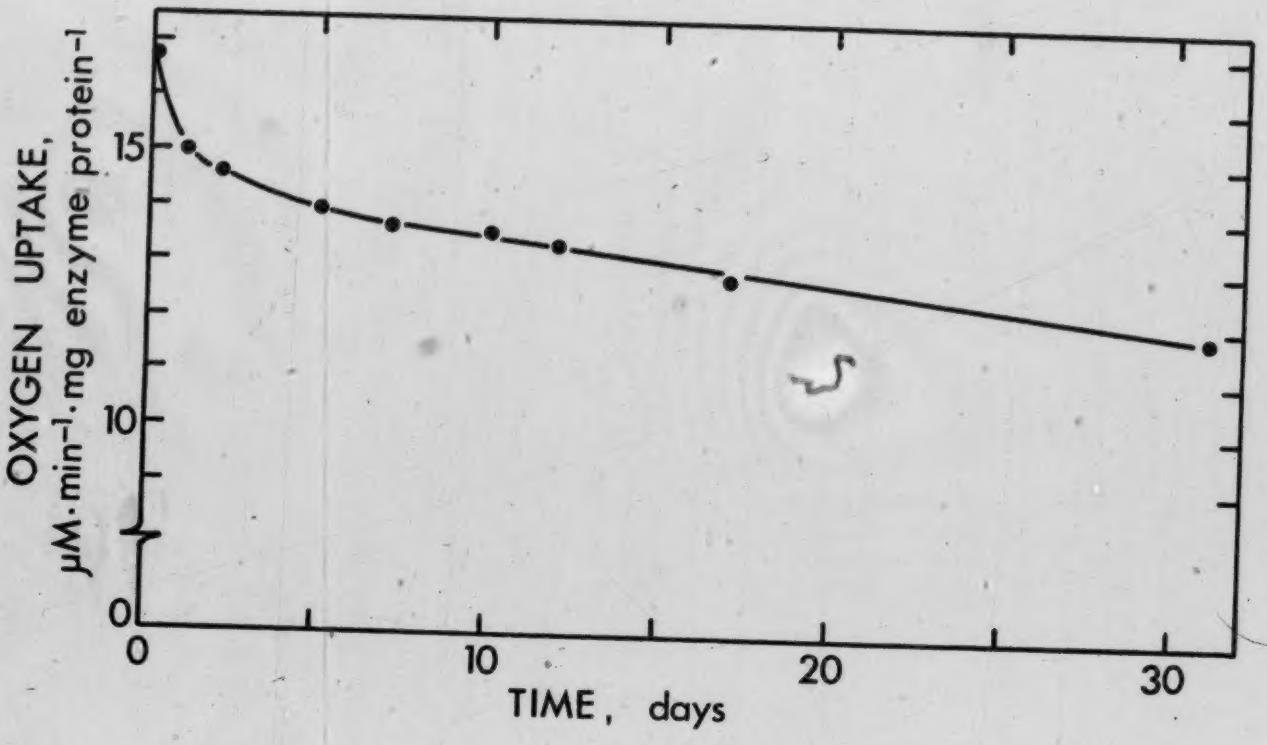


Figure 12. Activity of pea lipoxygenase as a function of storage time at 4°C. The enzyme preparation tested was contained in the first DEAE-cellulose column effluent peak obtained from Procedure III. The standard oxygraph assay, at 25°C., was performed on aliquots containing 12.5 μg protein (Lowry method).

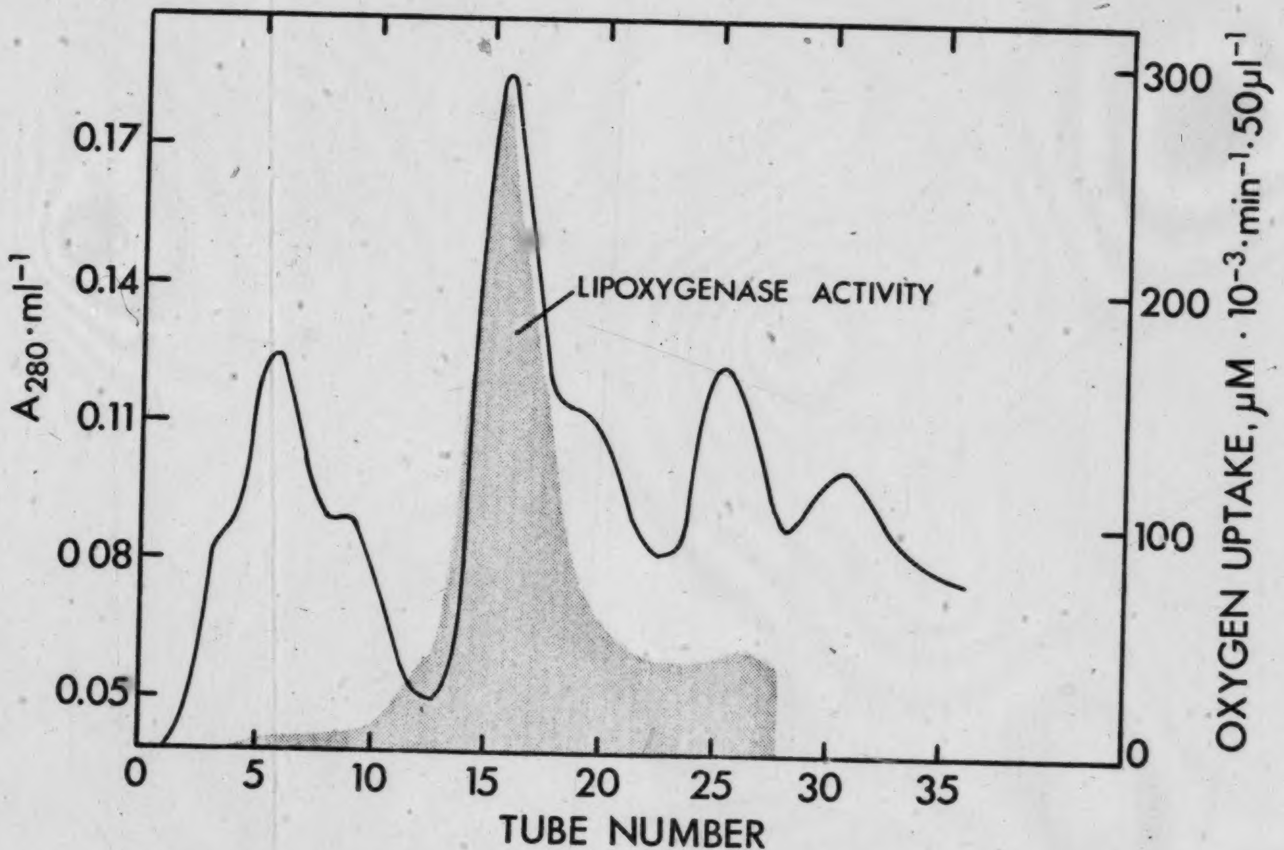


Figure 13. Pattern of A_{280} and pea lipoxygenase activity observed in DEAE-cellulose column effluent, Procedure III. Standard oxygraph assay at 25°C. Cf. Table 3.

Table 3. The Effect of Cyanide (5 mM) on Pea Lipoxygenase Activity*

Peak**	Procedure	% Inhibition
1	I	68
2		100
3		87
3		96***
1	III	88
2		80
3		100

* Based upon standard oxygraph assays at 25°C.

** Refers to A_{280} of DEAE-cellulose column effluent fractions.

*** The third peak was also tested with 5 mM Ca^{2+}.

Table 4. The Effects of Iron and Copper on Pea Lipoxygenase Activity

Substrate	O_2 depletion (%/min)* before addition of enzyme	O_2 depletion (%/min)* after addition of enzyme**	Inhibition (I) or stimulation (S) of enzyme with respect to standard substrate in absence of metal ion
Linoleic acid	0	98.0	
Linoleic acid + 50 μM Fe^{2+}	0	113.0	15.3% S
Linoleic acid + 50 mM Fe^{2+}	11.4	8.3	91.5% I 27.2% I***
Linoleic acid + 50 μM Fe^{3+}	0	112.0	14.3% S
Linoleic acid + 50 mM Fe^{3+}	25.0	25.0	74.5% I No effect***
Linoleic acid + 20 μM Cu^{2+}	0	4.7	95.2% I

* Barometer, 699 mm Hg, steady throughout.

** Samples of 50 μl from the first peak of the DEAE-cellulose column effluent, Procedure I, were used with the standard oxygraph assay at 25°C.

*** With respect to standard substrate in the presence of metal ion.

enzyme preparation contained in the first DEAE-cellulose column effluent peak, Procedure II, are presented in Figure 14. It is evident that pH optimum is at or near pH 7.0 for all three substrates tested. Trilinolein displayed the sharpest peak, falling off rapidly on either side of pH 7.0, but rising again above pH 8.5. Linoleic acid showed a broad peak, centered at pH 7.0 also. Methyl linoleate was seen to have the broadest peak, with its summit at pH 7.5 and a secondary rise (shoulder) at pH 8.5. Figure 14 reveals that the enzyme preparation was not very specific with regard to substrate. The oxygen uptake per min per mg of protein contained in enzyme preparation increased in the order methyl linoleate, linoleic acid, trilinolein in the most active pH region (pH 6.0 to pH 8.0). At pH 7.0, these values ($\mu\underline{M} \cdot min^{-1} \cdot mg^{-1}$) were 0.414, 0.887 and 1.041, respectively.

Dependence upon temperature of the activity of lipoxygenase from the first DEAE-cellulose column effluent peak, Procedure II, is shown in Figure 15. In Table 5, are given experimentally derived temperature coefficients. The greatest activity occurred at 30°C., whereas the greatest positive temperature coefficient was found to occur in the 10° - 15°C. range.

The heat stability of lipoxygenase from the first DEAE-cellulose column effluent peak, Procedure III, at the different temperatures described in Methods A.2, is

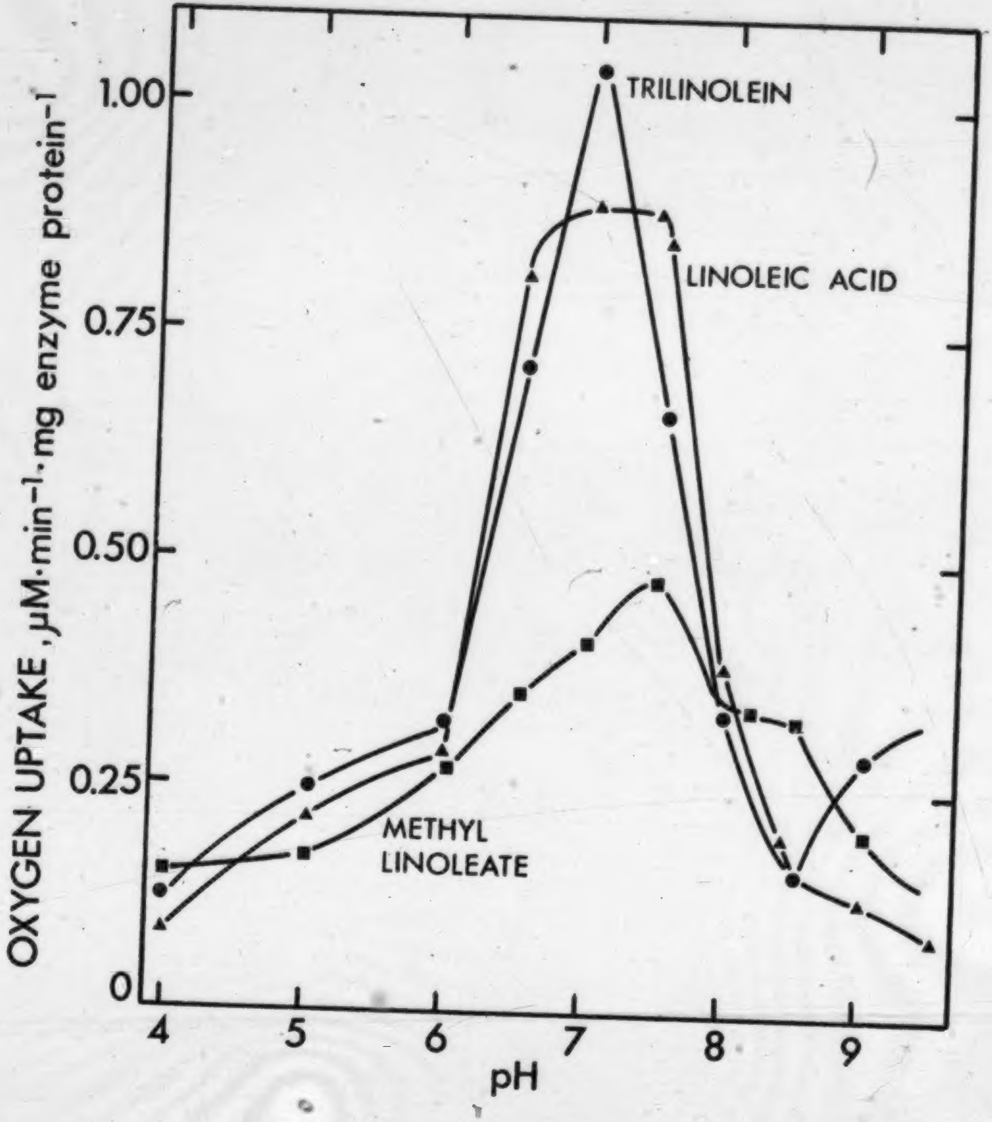


Figure 14. Activity of pea lipoxygenase as a function of pH and substrate. Enzyme preparation was from first DEAE-cellulose column effluent peak, Procedure II.

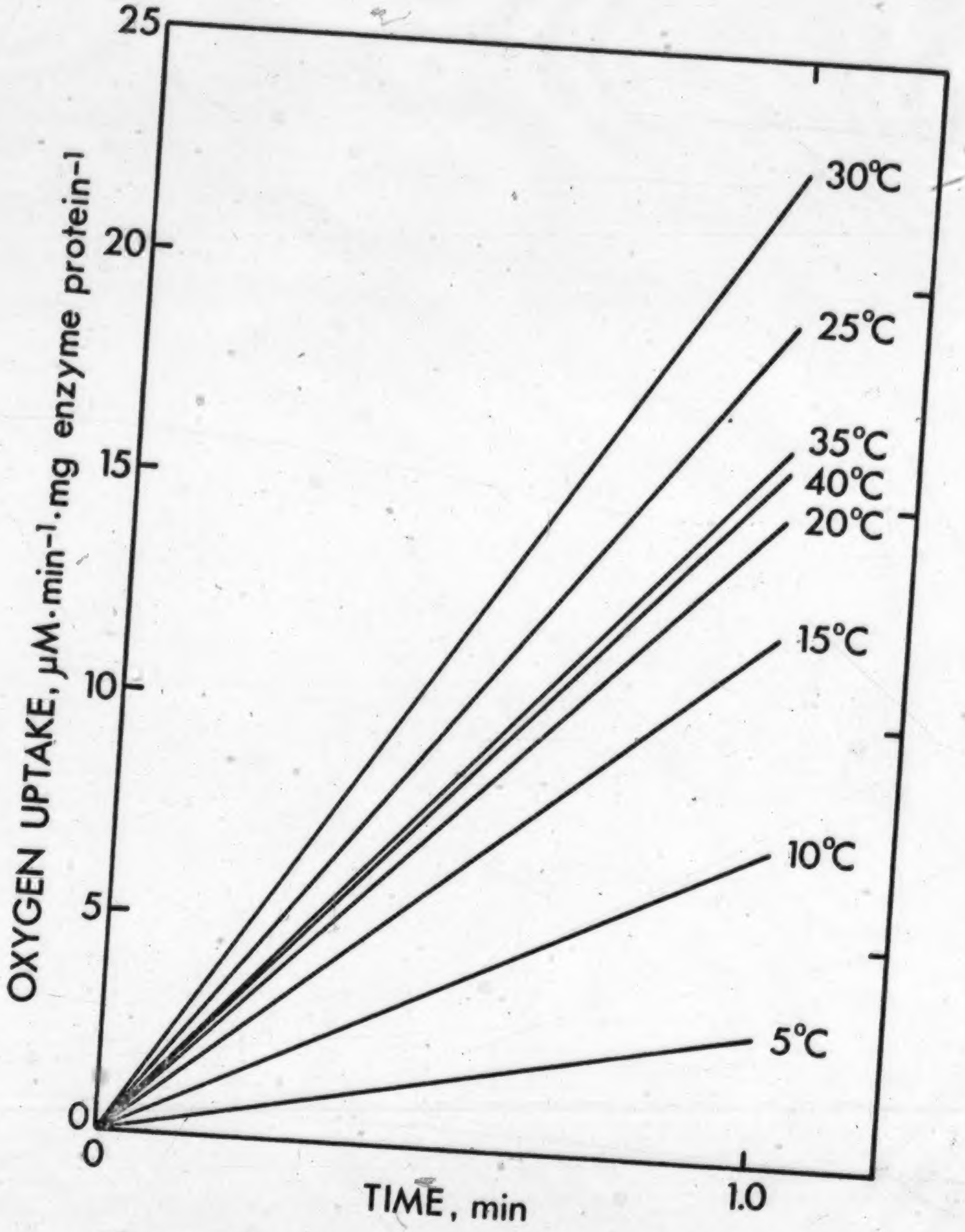


Figure 15. The effect of temperature on pea lipoxygenase activity. Enzyme preparation was from the first DEAE-cellulose column effluent peak , Procedure II. Standard oxygraph assays were performed at indicated temperatures.

Table 5. Pea Lipoxygenase Temperature Coefficients*

Temperature (°C)	O_2 uptake, $\mu\underline{M}.min^{-1}$ $\cdot$ mg enzyme protein^{-1}	ΔO_2 uptake over 5°C.	Temperature Coefficient	
			Range (°C)	ΔO_2 uptake, $\mu\underline{M}.min^{-1}$.mg enzyme protein^{-1} $\cdot$ °C.$^{-1}$
5°	2.86			
10°	7.11	4.25	5°-10°	+0.85
15°	12.36	5.25	10°-15°	+1.05
20°	14.88	2.52	15°-20°	+0.504
25°	18.94	4.06	20°-25°	+0.812
30°	22.59	3.65	25°-30°	+0.73
35°	16.20	6.39	30°-35°	-1.278
40°	15.86	0.34	35°-40°	-0.068

* Based on standard oxygraph assays in which 50 µl aliquots of DEAE-cellulose column effluent (first peak, Procedure II), containing 20.2 µg protein, were used.

summarized in Figure 16 and Table 6. Not shown in Figure 16 are the results of inactivation at 80°C. and 90°C. At these latter two temperatures, inactivation was complete after one minute; that is, all oxygraph assays recorded zero oxygen uptake. As Table 6 shows, total inactivation occurred also at 70°C. after 20 min, heat stability is just under 50% after 20 min at 60°C. and just over 2/3 after 20 min at 50°C. The data indicate, also, that heating at 60°C. for one minute is equivalent to 10 min at 50°C., both reducing activity to 75.6% of the original.

Results of molecular weight determinations of lipoxygenases from pea and soybean, and of pea albumins and globulins, are reproduced in Figures 17, 18 and 19.

Figure 17 is typical of results obtained with several batches of SDS gels in which good straight lines were realized with the six standard proteins employed (see Evaluation of Calibration Curve Used in Determination of Molecular Weights, Appendix). The pea lipoxygenase, which was contained in an aliquot from the first peak of DEAE-cellulose column effluent (Procedure I), yielded no fewer than four bands (cf. Figure 19; only three of these are located on the calibration curve, Figure 17). Six bands were obtained from the commercial soybean lipoxygenase preparation (Figure 18). The first band for the pea and soybean enzyme preparations (corresponding to molecular weights of 106,000 and 106,500, respectively) proved to

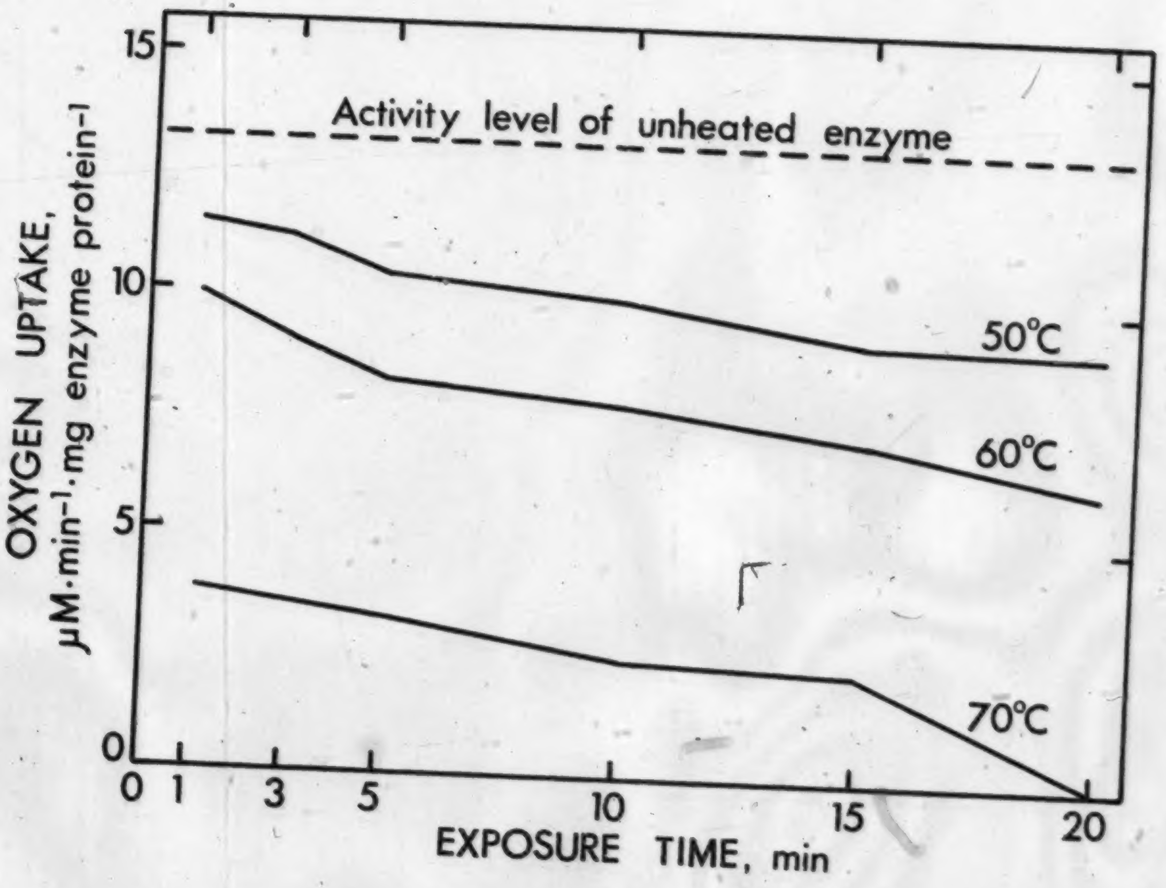


Figure 16. Stability of pea lipoxygenase at temperatures used for blanching. For description see Methods A.2.

Table 6. Heat Stability of Pea Lipoxygenase Held at Different Temperatures For Times Indicated.*

°C \ Min	1	3	5	10	15	20
50	86.5	85.0	78.2	75.6	69.5	67.7
60	75.6	66.9	62.4	58.3	51.5	47.4
70	28.2	25.9	24.4	19.5	16.9	0
80	0	0	0	0	0	0
90	0	0	0	0	0	0

* Expressed as % of activity of unheated enzyme. For details see Methods A.2.

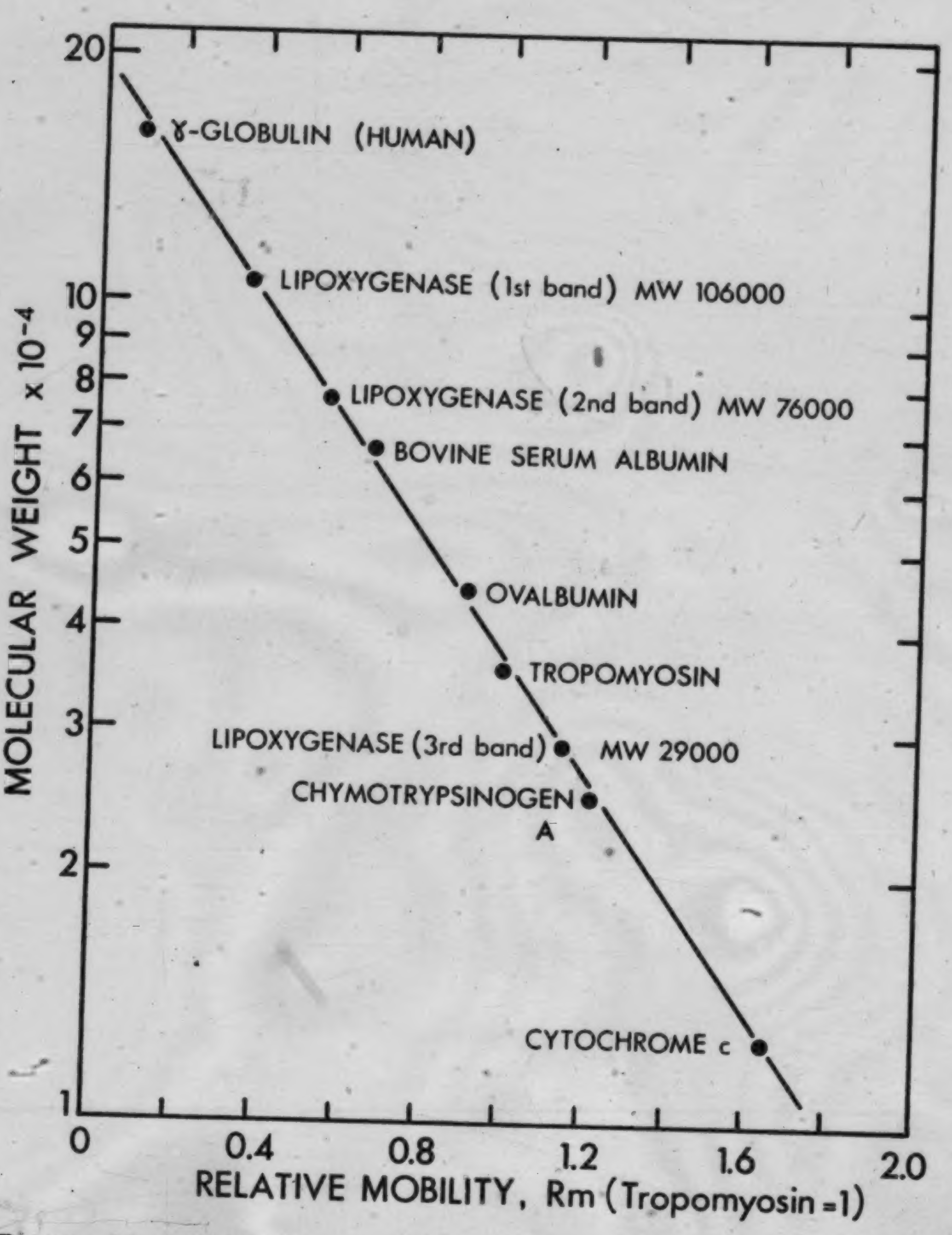


Figure 17. Calibration curve used for determination of molecular weights of pea lipoxygenase. Preparation of enzyme according to Procedure I. Cf. pea seed enzyme data, Figure 18. The equation for the line of best fit drawn through the data points is log y = mx + b, where m = 0.73588, b = 5.29579, y = molecular weight and x = distance of migration relative to tropomyosin.

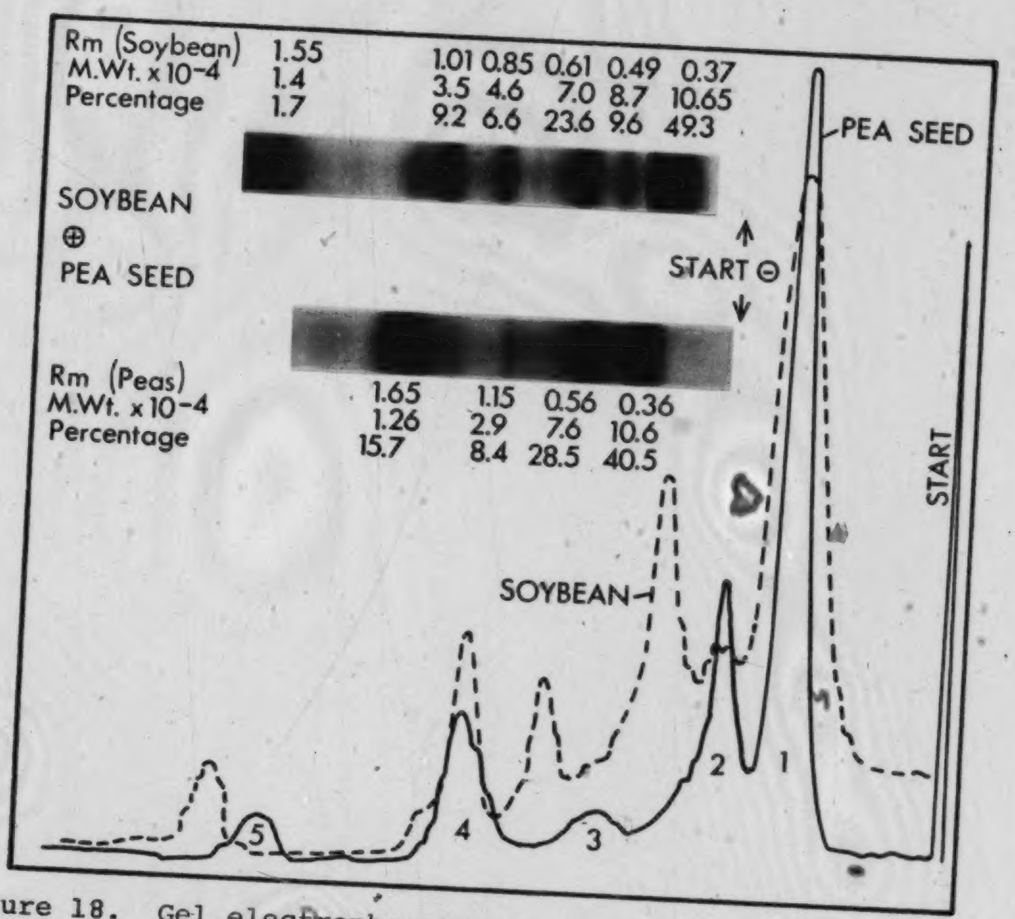


Figure 18. Gel electrophoretograms of purified pea seed and commercial soybean lipoxygenases and their scanning patterns. The two gels were run in separate batches and their relative mobilities (Rm) are not comparable. Figure 17 is the calibration curve used for the pea seed lipoxygenase.

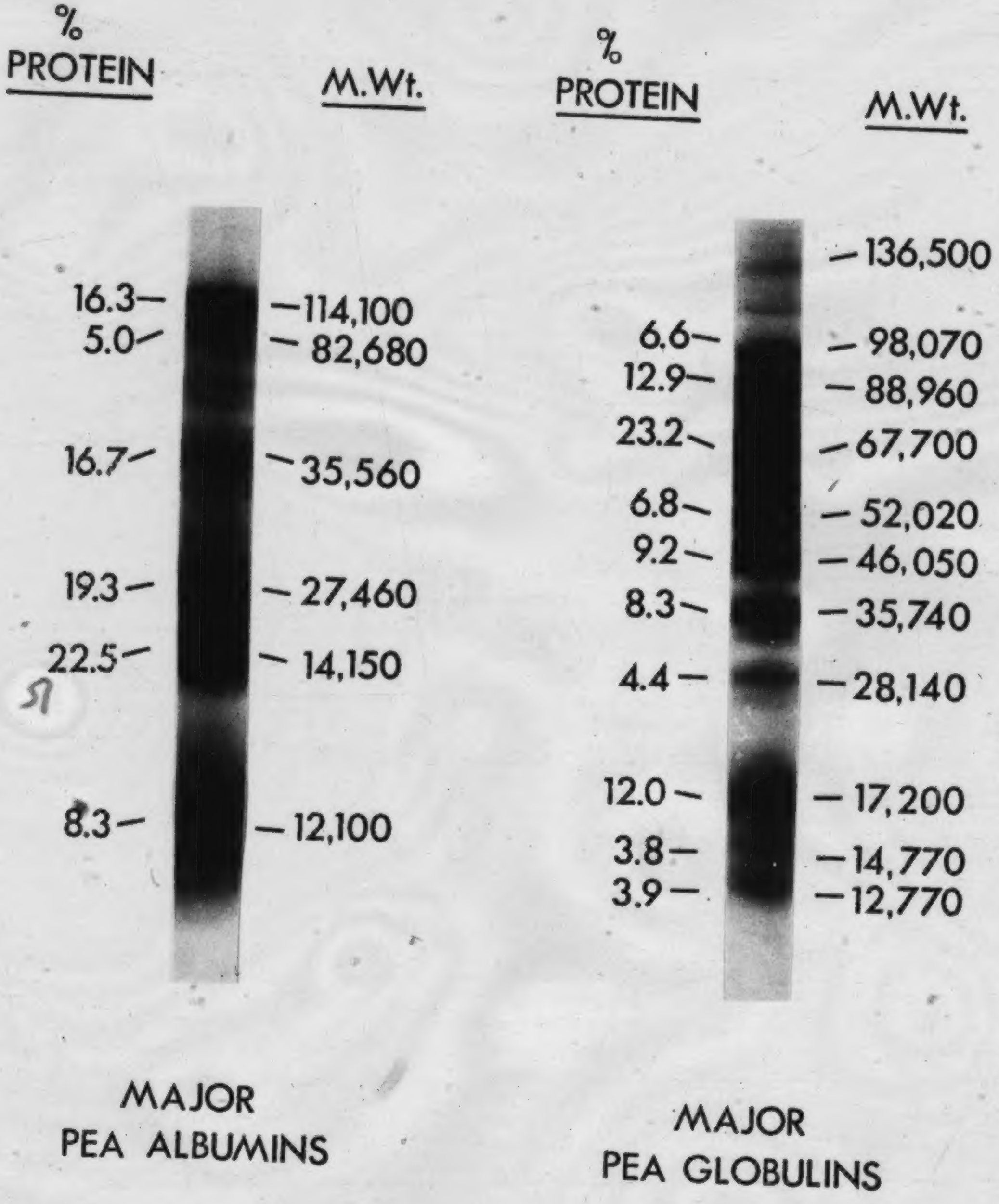


Figure 19. Gel electrophoretograms of major pea seed albumins and globulins. For isolation procedures, see Figure 9; molecular weights were determined as for lipoxygenases.

be, in each case, the major constituent (40.5% and 49.3%, respectively).

In Figure 19, only the major bands of the pea albumins and globulins are depicted, six for the former and eleven for the latter (the globulin band exhibiting a molecular weight of 136,500 was present only to the extent of about 2.5%). Actually, there were 20 and 25 bands discernible, respectively, but minor bands (present in quantities of only a few percent or less) were not labelled in Figure 19.

Zymograms of lipoxygenases from fresh green peas and from pea seed four months, six months and one year old are reproduced in Figure 20 (vertical axis to scale, 1:1). It is noted that the second band becomes weaker with age and disappears after one year. A zymogram of commercial soybean lipoxygenase is included for comparison. When comparing Figure 20 with Figures 17 and 18, one can deduce that the active enzyme components of lipoxygenase (isoenzymes) are fewer in number than the protein components visualized in the SDS gels. Pea lipoxygenase thus contains at least four distinct protein components on SDS gels, but only two (if fresh), or one (if one year old), enzymically active components on zymogram. Similarly, the commercial soybean lipoxygenase, which displays six protein components on SDS gels, has only one zymogram band. Zymogram bands depicted in Figure 20 were not affected by

Fig. 20

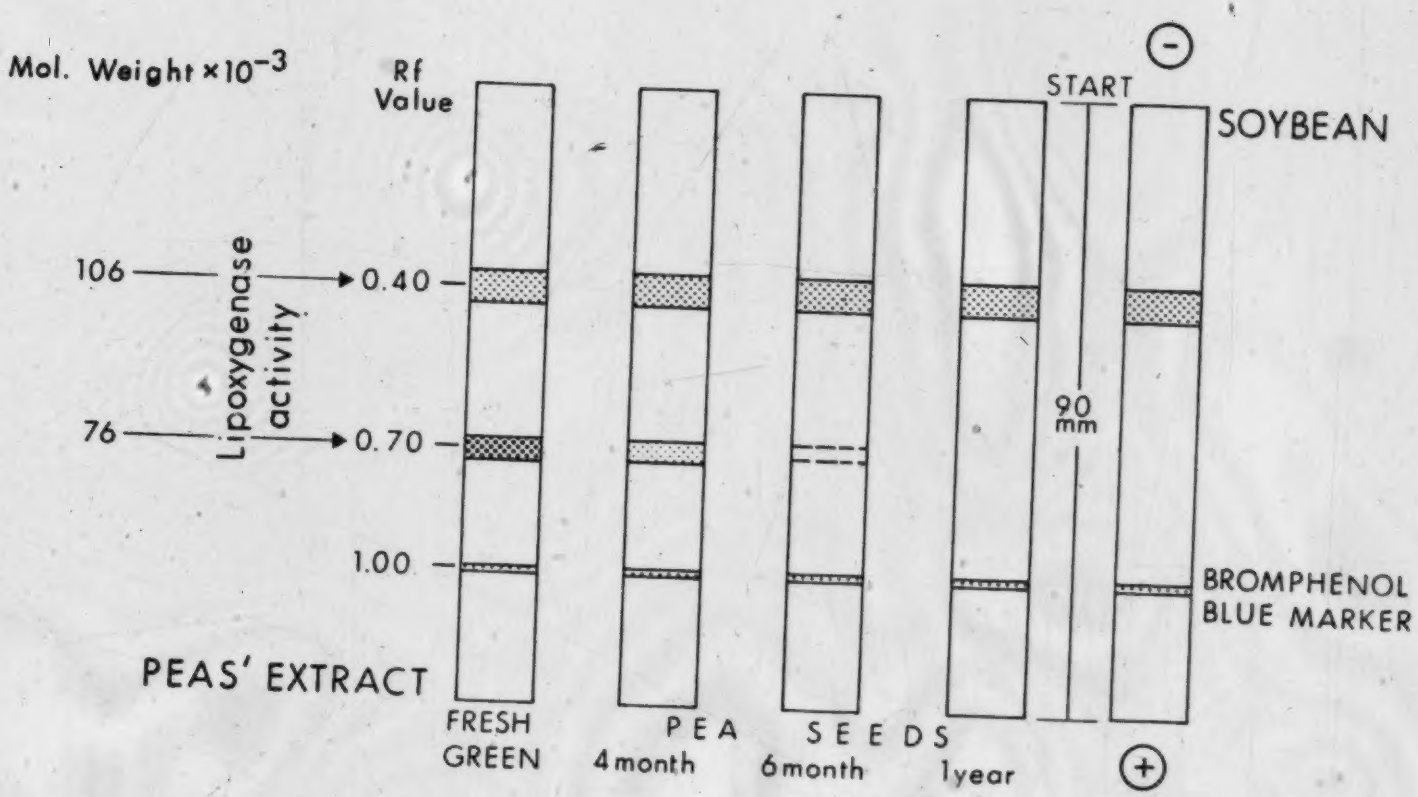


Figure 20. Isoenzyme patterns of purified lipoxygenase isolated from fresh green peas and pea seeds. A zymogram of commercial soybean lipoxygenase is included for comparison. The weak, residual enzyme activity observed at the top of the gels is not illustrated.

addition of 5 mM cyanide to the substrate solution before staining.

2. Enzymic Destruction of β-carotene.

Comparison of the efficacy of lipoxygenase from the pea (first DEAE-cellulose column effluent peak, Procedure I) in the β-carotene bleaching assay with that of soybean lipoxygenase, at 230 μg · ml^{-1} and at 10 μg · ml^{-1}, is made in Figure 21 and Table 7. This preliminary study established that the lower level of concentration for each enzyme was adequate for the proposed 15-min studies and that relatively large amounts of enzyme preparations were not required to obtain significantly measurable results in short periods of time. Furthermore, rapid pigment depletion, as occasioned, for example, by the higher concentration, could be avoided (see Table 7).

Figures 22 through 26 and Tables 8 through 10 include all the data derived from the enzymic β-carotene bleaching work at the five different pigment levels.

In Figures 22 and 23, it is seen that the intermediate β-carotene concentration, c, afforded the most rapid pigment destruction, the curves in each case rising the most abruptly from zero time. The incremental plots of β-carotene destruction (Figure 24) reveal this initial rate of reaction in the c mixtures. When pea lipoxygenase was used (Figure 22), depletion of β-carotene was complete in reaction mixture a after 2 1/4 min, in b after 7 1/2

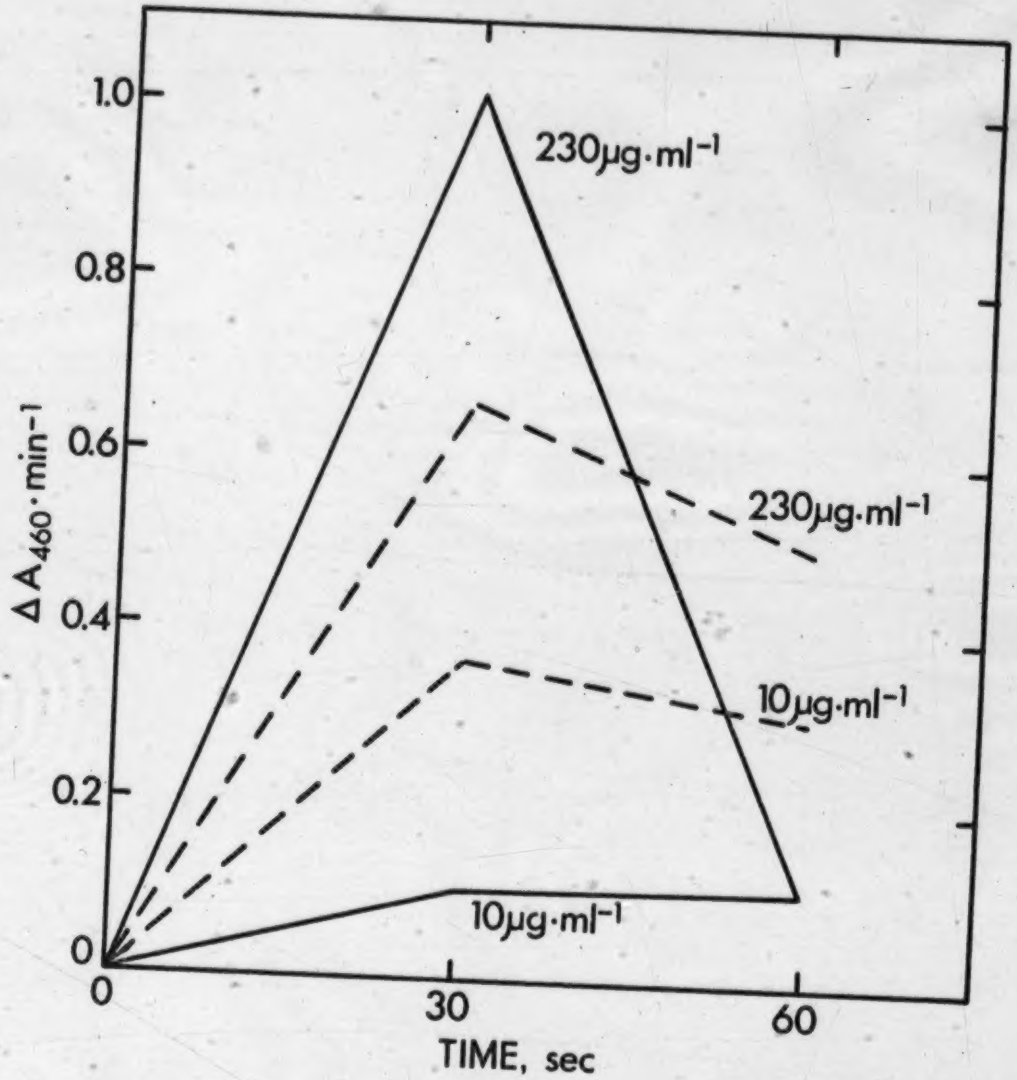


Figure 21. Destruction of β-carotene by lipoxygenases in reaction mixture c at 25°C. Legend: pea enzyme (———), soybean enzyme (---). See Methods A.3.

Table 7. Preliminary Study: Beta-Carotene Destruction Occurring in Reaction Mixture c in First 30 Seconds, Expressed as % of Initial Concentration of Pigment*

Enzyme concentration	Pea lipoxygenase	Soybean lipoxygenase
230 $\mu g \cdot ml^{-1}$	78.3	76.7
10 $\mu g \cdot ml^{-1}$	8.1	30.0

---

* Assay at 25°C.

min, in c after 9 1/2 min, and in d and e, not at all after 15 min. The rate of depletion in d was greater than in e, as seen by the fact that 79.1% of the β-carotene in the former, and 57.8% in the latter, had been destroyed after 15 min (cf. the two horizontal columns at the foot of Table 8). The courses of all five reactions graphed in Figure 22 indicate a maximum rate initially, with diminution of rate setting in sooner or later. By contrast, soybean lipoxygenase under the same conditions (Figure 23) displayed a different order of depletion of pigment: first in a, then in c, b, d and e. Moreover, maximum rate was attained initially only in a, b and c. After 15 seconds, a steady state was maintained in reaction mixture d (the curve became a perfect straight line) until depletion of β-carotene, the initial rate being less. In e (Figure 23), the initial rate of β-carotene destruction was minimal, increasing slightly but steadily throughout the 15 min. Likewise, e of Figure 22 approached linearity throughout, but with a slight downward skew. The e sections of Figure 24 reveal these gradual changes graphically.

Total amounts of β-carotene destroyed (μg · mg enzyme protein^{-1}) in each one-minute interval are given in Table 8 for the different systems tested. These incremental values give essentially the same kind of information found in Figure 24, but provide the reader with numbers. It is clear that within 15 min all initial pigment

Table 8. Destruction Rate* of Beta-Carotene for Each Minute

Minute	Pea Lipoxygenase in Reaction Mixture					Soybean Lipoxygenase in Reaction Mixture				
	a	b	c	d	e	a	b	c	d	e
1	144.5	169.3	365.1	110.0	132.4	188.0	339.0	621.3	199.0	27.6
2	27.2	76.1	88.0	110.0	99.2		23.2	110.8	249.7	20.7
3	15.7	41.4	72.5	110.0	77.2		8.1	17.8	276.1	25.3
4		24.2	53.1	82.4	88.4		4.7		251.7	60.1
5		19.1	49.2	110.0	88.4				239.6	39.2
6		20.8	35.1	96.2	88.4				284.2	50.9
7		8.6	27.2	73.3	88.4					34.5
8		15.5	31.2	80.0	88.4					62.4
9			18.2	75.5	88.4					53.3
10			10.4	61.7	88.4					67.1
11				66.4	88.4					67.1
12				54.9	77.2					67.1
13				57.1	59.5					67.1
14				52.6	77.2					69.4
15				45.7	71.6					78.6
$\sum$	187.4	375.0	750.0	1185.8	1301.5	188.0	375.0	749.9	1500.3	790.4
**	187.5	375	750	1500	2250	187.5	375	750	1500	2250

* As µg · mg enzyme protein^{-1} · min^{-1}; for details see Methods A.3.

** β-carotene initially present (µg · mg enzyme protein^{-1}).

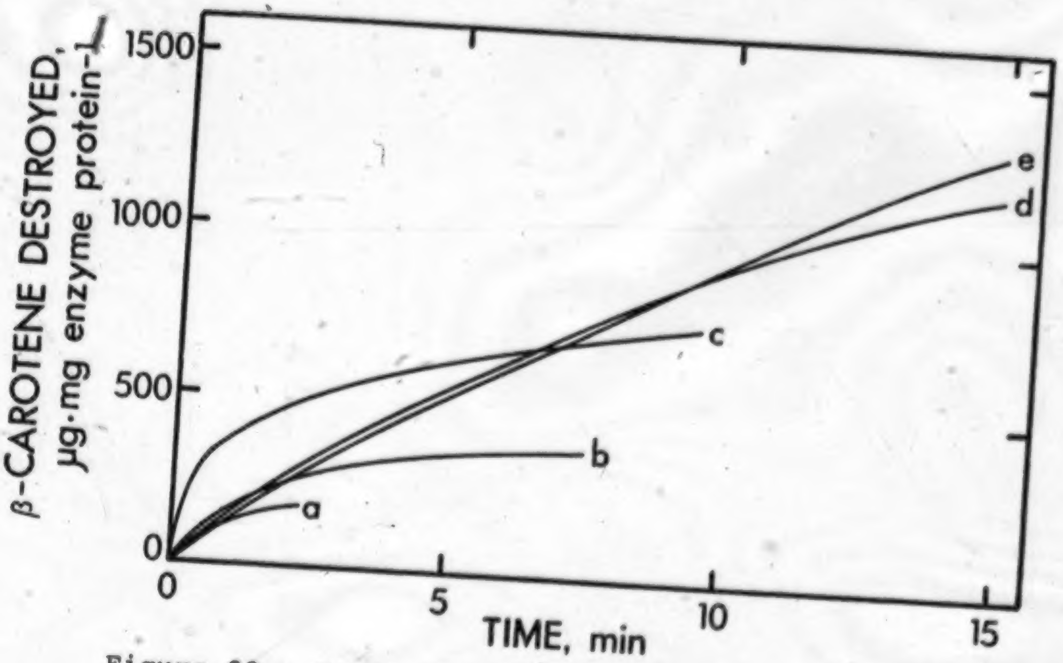


Figure 22. Cumulative β-carotene destruction in 15 min in the presence of linoleic acid and pea lipoxygenase.

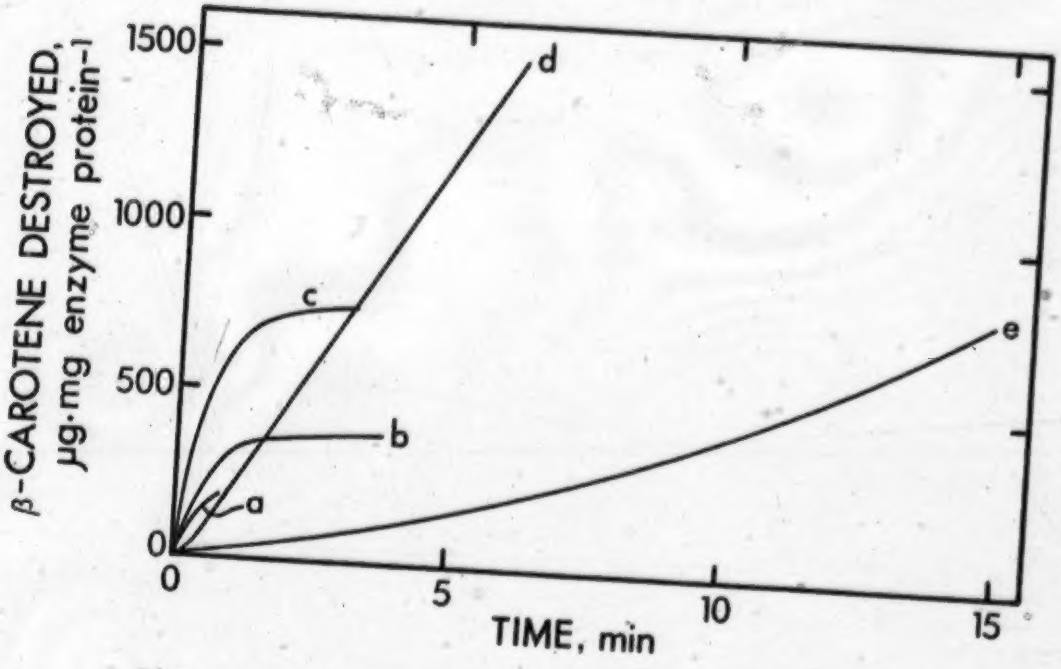


Figure 23. Cumulative β-carotene destruction in 15 min in the presence of linoleic acid and soybean lipoxygenase.

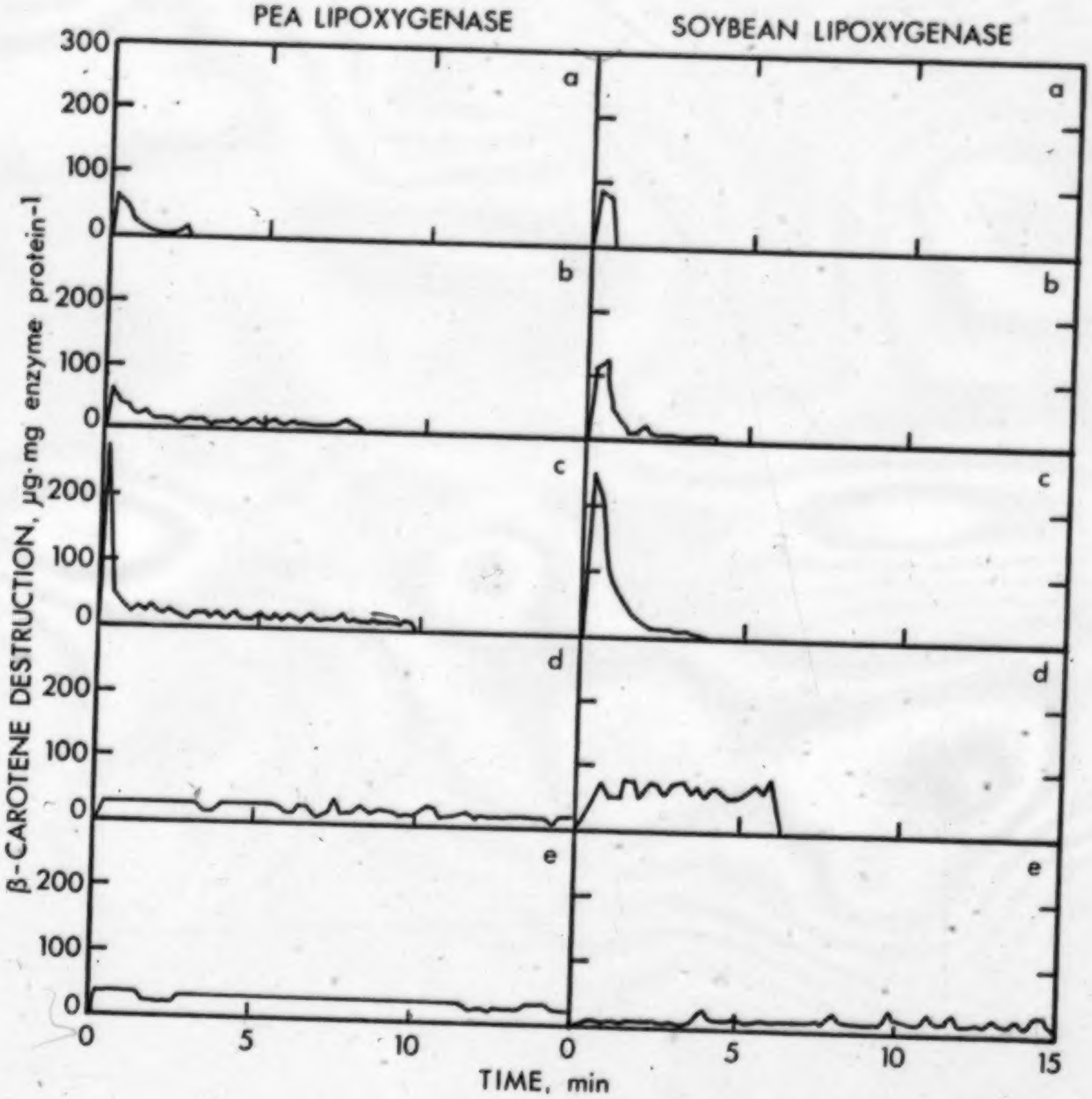


Figure 24. Incremental β-carotene destruction, mediated by lipoxygenases from the pea and from soybean, in the presence of linoleic acid. Measurements were made at 15-sec intervals over 15 min. For initial β-carotene concentrations a through e and other details, see Methods A.3.

was destroyed by pea lipoxygenase in reaction mixtures a, b and c, but not in d and e, and that the soybean lipoxygenase was more active, since all pigment was destroyed in a, b, c and d, under the same conditions, and only in e was destruction incomplete in 15 min.

Table 9 compares initial rates of destruction of β-carotene in the different systems. Apparent only are general trends of increase with increasing pigment up to reaction mixture c, and then a falling off, with no evident quantitative correlations. The values for d and e in the pea enzyme system reverse this pattern.

Figures 25 and 26 compare initial oxygen uptake values (solid lines) with initial β-carotene destruction rates (broken lines) for bleaching systems catalyzed by pea and soybean lipoxygenases, respectively. In the former, the oxygen uptake was little influenced by the amount of pigment in the system, although, compared to control containing no pigment, the presence of β-carotene in all five concentrations reduced uptake of oxygen by about 70%. An entirely different pattern is seen in Figure 26. First, oxygen uptake of the control, which contained no pigment, was 6.05 times greater than that observed when pea lipoxygenase was used. Then, a varied pattern of oxygen uptake was observed. At the lowest concentration of β-carotene, reaction mixture a, oxygen uptake was elevated 15.0% above the control, at b oxygen uptake was nearly the same as the

Table 9. Initial Rates* of Destruction of Beta-Carotene in Different Bleaching Systems.

Reaction	Relative Concentrations	Pea Lipoxygenase	Soybean Lipoxygenase
a	0.25	58.7	94.0
b	0.5	62.2	117.3
c	1	277.1	243.7
d	2	27.5	24.4 or 65.1**
e	3	33.1	6.9

* As µg · mg enzyme protein^{-1} · 15 sec^{-1}; for details see Methods A.3.

** Disregarding lag period.

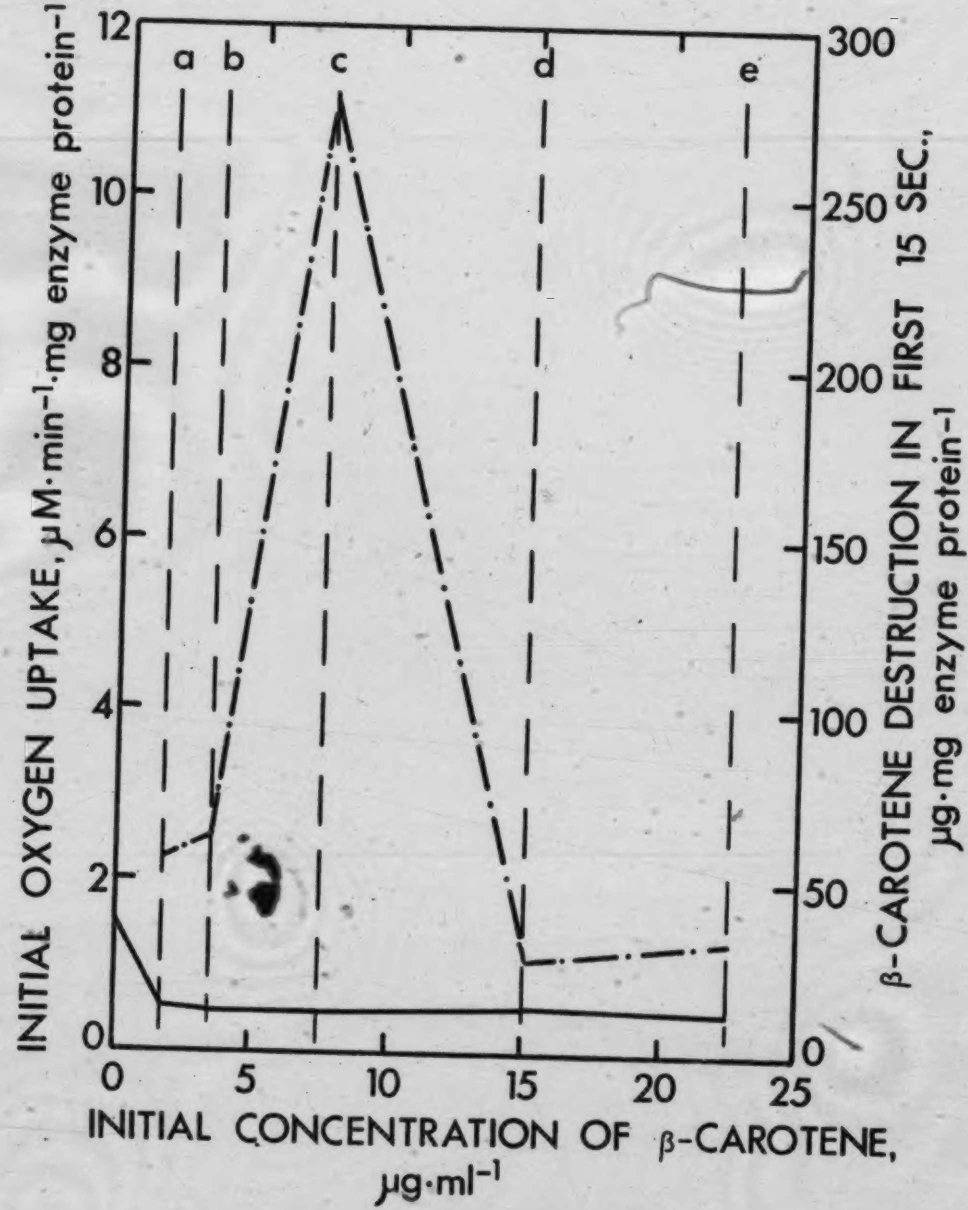


Figure 25. Comparison of initial O_2 uptake with initial pigment destruction in β-carotene bleaching systems containing pea lipoxygenase. Legend: oxygen uptake (——); pigment destruction (-·-·-).

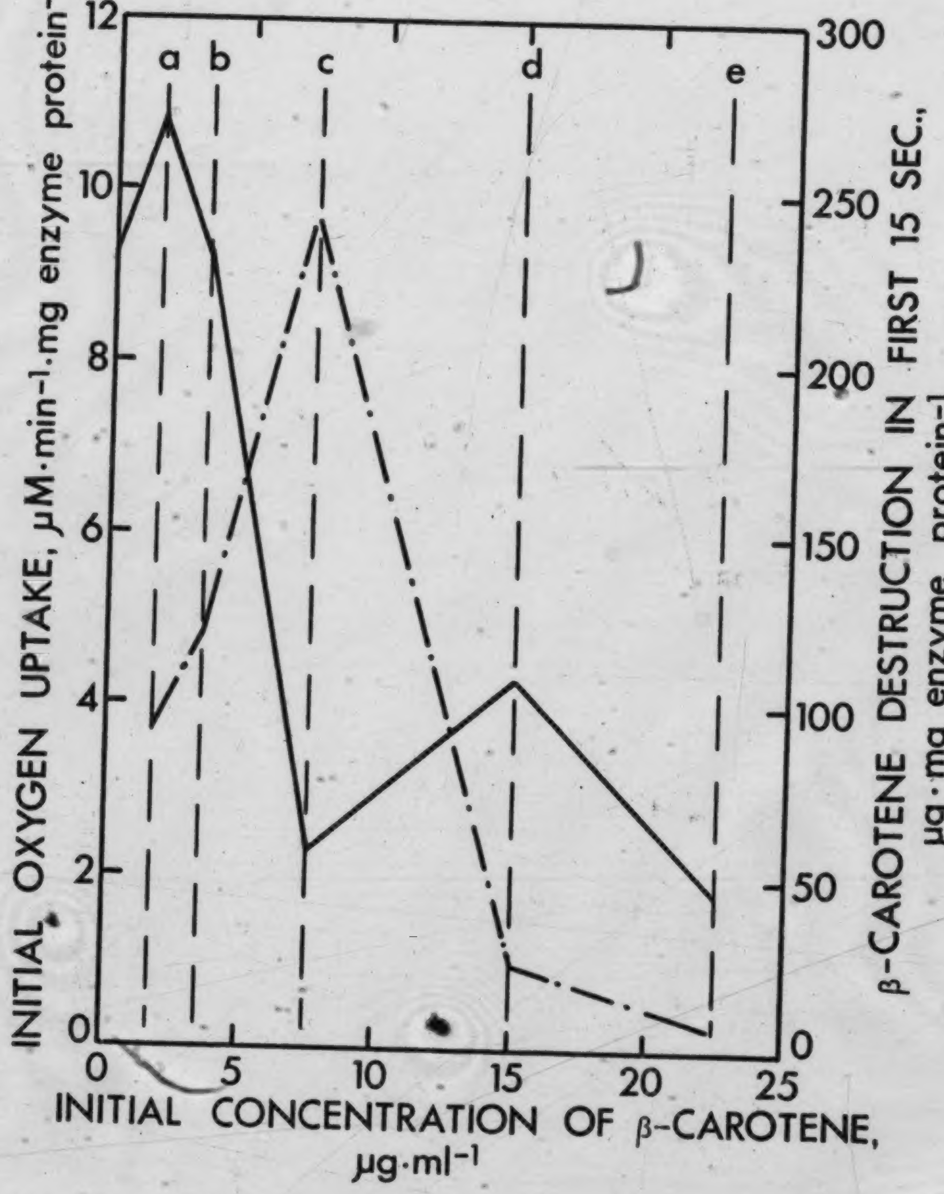


Figure 26. Comparison of initial O_2 uptake with initial pigment destruction in β-carotene bleaching systems containing soybean lipoxygenase. Legend: oxygen uptake (———); pigment destruction (-·-·-·).

control (<0.2% lower), at c where pigment destruction was greatest, oxygen uptake was 74.6% below that of the control, at d oxygen uptake was down 53.8% from the control, and at e, where pigment destruction was lowest, so was oxygen uptake, at 80.5% less than control. Table 10 summarizes these relationships.

B. Non-enzymic Oxidations.

The results of the preliminary oxygen uptake experiment in the dark, using dry filter paper impregnated with linoleic acid + crocin or linoleic acid + β-carotene, are summarized in Figure 27. Linoleic acid was partially protected from oxidation by the presence of crocin, to approximately the same extent, by all three concentrations of the pigment employed (10^{-8}, 10^{-6} and 10^{-4} M). In the presence of β-carotene, however, linoleic acid was protected only at the lowest concentration employed (10^{-8} M) and only very slightly. At the higher concentrations (10^{-6} and 10^{-4} M) β-carotene enhanced oxygen uptake.

The data from a parallel oxygraph experiment, in which the constituents were in solution in the presence and in the absence of soybean lipoxygenase, are presented in Figure 28. Enzymically catalyzed oxidation of linoleic acid in solution (solid line curves) was evidently unaffected by the pigments at the lower concentrations (10^{-8} M crocin, 10^{-8} and 10^{-6} M β-carotene). Crocin was protective at the higher concentrations (10^{-6}, 10^{-4} and

Table 10. Comparison of Initial Oxygen Uptake With Initial Destruction of Beta - Carotene in Different Bleaching Systems*

Enzyme	Reaction Mixture	Oxygen Uptake	Δ**	β-carotene Destruction
	Control	1.537		
Pea Lipoxygenase	a	0.500	- 67.5%	58.7
	b	0.420	- 72.6%	62.2
	c	0.420	- 72.6%	277.1
	d	0.520	- 66.2%	27.5
	e	0.497	- 67.7%	33.1
	Control***	9.297		
Soybean	a	10.693	+ 15.0%	94.0
	b	9.280	- 0.18%	117.3
	c	2.357	- 74.6%	243.7
	d	4.303	- 53.8%	24.4
	e	1.813	- 80.5%	6.9

* Units as in Figures 25 and 26.

** As % change from oxygen uptake of control, which lacked pigment.

*** Ratio of control values, 9.297 to 1.537, = 6.05.

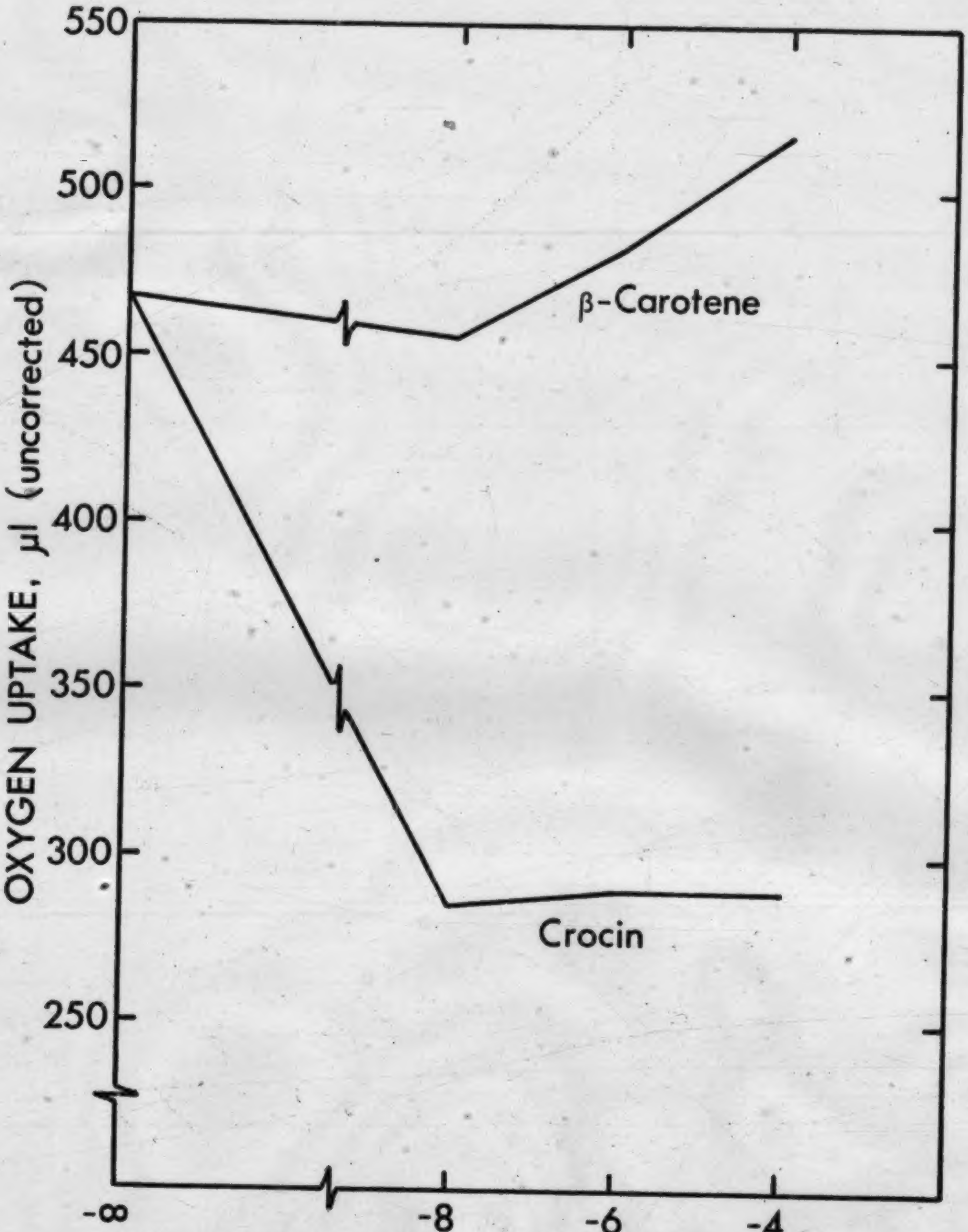


Figure 27. Total non-enzymic oxygen uptake after 5 h in the dark by dry filter paper matrices impregnated with lineoleic acid and variable amounts of carotenoid. Linoleic acid level was constant. The experiment was performed in Gilson respirometer Model GR 20 at 25°C. See Methods B.1.a. for experimental details.

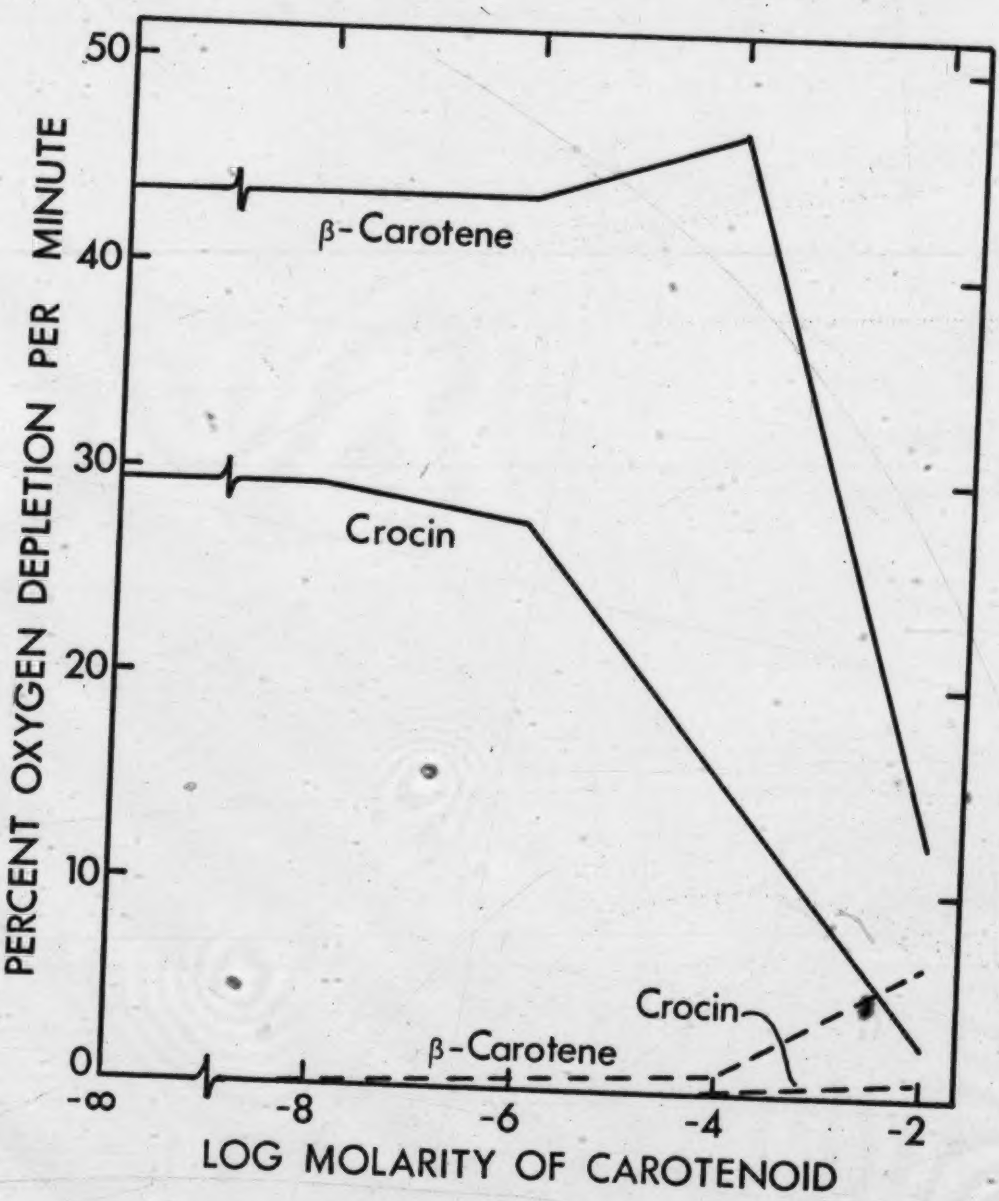


Figure 28. Initial rate of enzymic and non-enzymic consumption of oxygen in the dark as measured in the oxygraph cell containing linoleic acid and variable amounts of carotenoid. Enzyme used, soybean lipoxygenase; temperature, 25°C. See Methods B.1.a. for other details.

10^{-2} $\underline{M}$) in the enzymic reaction, whereas β-carotene stimulated the reaction when present to the extent of 10^{-4} $\underline{M}$, but was antioxidative at the still higher concentration of 10^{-2} $\underline{M}$ (very near the maximum solubility under the prevailing conditions). The broken line curves in Figure 28 indicate the pro-oxidative influence of the pigments in solution at the higher concentrations in the absence of enzyme.

Data from the preliminary experiments concerned with the effect of wheat proteins upon non-enzymic oxidation of linoleic acid are presented in Figure 29 and Table 11. They were carried out in the dark at different temperatures and include β-carotene as the parameter of particular interest.

It is seen that the curves representing matrices alone (without linoleic acid) have similar shapes and positions, within a temperature category, regardless of matrix. In all four experiments (Figure 29a, b, c and d) these curves indicate the least oxygen uptake, or the most net gaseous evolution. When comparing results from experiments performed at 25°C. (Figure 29a) with those obtained at 40°C. (Figure 29b), it is evident that, in the former, oxygen uptake was sluggish and that relative positions of curves are not consistent with those of the latter. Nor are the relative positions of curves in a or b consistent with those obtained from experiments conducted at

Figure 29. Volume changes in dry Gibson respirometer flasks, containing one cellulose or wheat protein matrix disc impregnated with linoleic acid ± β-carotene, and maintained at constant pressure in the dark for 50 h. Positive values indicate volume decrease (apparent O_2 uptake); negative values indicate volume increase (net gaseous evolution). Plotted points are based on multiple values as indicated.

a. Park variety wheat proteins; 25°C.; duplicate; 10 points plotted.

b. Park variety wheat proteins; 40°C.; duplicate; 9 points plotted (experiment terminated at 39 h due to instrument failure).

c. Park variety wheat proteins; 50°C.; triplicate; 15 points plotted.

d. Thatcher variety wheat proteins; 50°C.; triplicate; 15 points plotted.

Abbreviations:

Cel = cellulose (filter paper)

Glu = glutenins

Glo = globulins

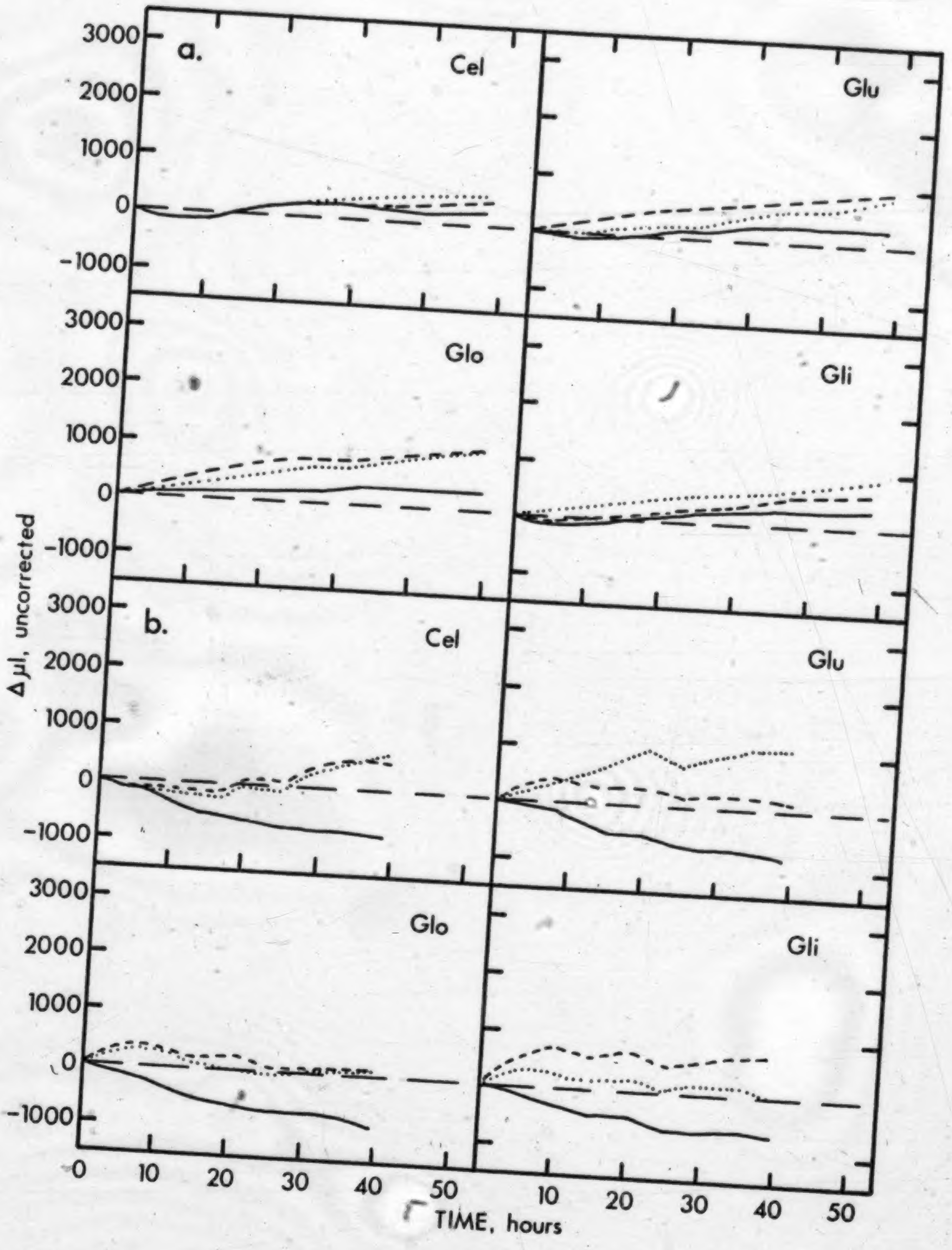
Gli = gliadins

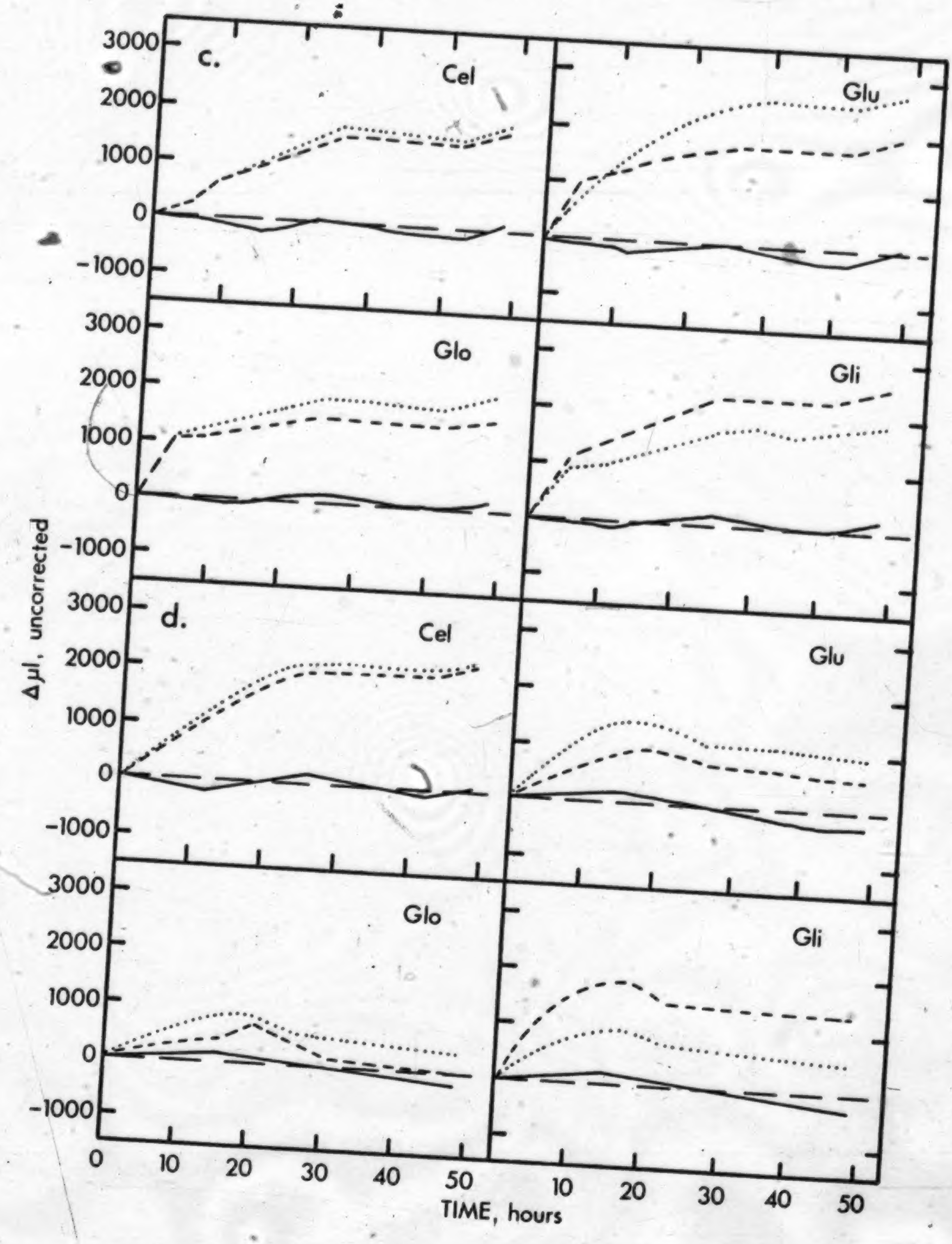
Legend:

———— Matrix alone (cellulose or wheat protein)

-------- Matrix impregnated with linoleic acid

.... Matrix impregnated with linoleic acid + β-carotene


a.
Cel
Glu
Glo
Gli
b.
Cel
Glu
Glo
Gli
3000
2000
1000
0
-1000
Δ µl, uncorrected
0
10
20
30
40
50
TIME, hours


c.
Cel
Glu
Glo
Gli
d.
Cel
Glu
Glo
Gli
3000
2000
1000
0
-1000
Δμl, uncorrected
0
10
20
30
40
50
TIME, hours

50°C. (Figure 29c and d). Also, at 40°C.(Figure 29b), there was a considerable net increase of gas phase within the flasks containing matrices lacking linoleic acid. Figures 29c and d reveal the more vigorous oxidations at 50°C. and the results, based on triplicate values, are straightforward. With regard to relative positions, the curves in c are consistent with those in d (50°C. was adopted for all subsequent work).

Comparison of total oxygen consumption recorded for a given category in Figure 29c with the same category in Figure 29d discloses substantial quantitative differences, the values in the former being consistently higher (see Table 11). One can accept the category of matrix alone (control) as indifferent, or zero uptake ± slight variations. In Figure 29c, as in Figure 29d, it is evident that the matrix + linoleic acid curve is lowest when the matrix is globulin. The greatest difference between oxygen uptake curves, when comparing matrix + linoleic acid categories of c (Park) with d (Thatcher), is found where the matrix consisted of globulins (Table 11). The essential information yielded by the wheat protein matrix experiments is that oxidation of linoleic acid is accelerated in the presence of β-carotene when the matrix is cellulose, glutenins or globulins and that the oxidation is inhibited by β-carotene when gliadins are used.

Experimental oxidations of pea lipids combined with

Table 11. Summary of Results of Preliminary Non-Enzymic Oxidation Experiments With Wheat Protein Matrices: Comparison of Effect of Wheat Variety Upon Total Oxygen Uptake*

	Park Variety Wheat Proteins (see Fig.29c).	Thatcher Variety Wheat Proteins (see Fig.29d).	Δ
Glutenins + linoleic acid	2110	600	1510
Glutenins + linoleic acid + β-carotene	2891	942	1949
Globulins + linoleic acid	1705	2	1703
Globulins + linoleic acid + β-carotene	2111	350	1761
Gliadins + linoleic acid	2606	1338	1268
Gliadins + linoleic acid + β-carotene	1926	542	1384

* As µl of ambient air, uncorrected, after 50 h at 50°C. in the dark.

dry matrices of pea carbohydrate and protein constituents, conducted at 50°C. in the dark and in intense light, constituted the major part of the non-enzymic work. The data from these experiments are summarized in Figures 30 and 31 and Table 12.

A study of Figure 30 reveals many aspects of non-enzymic oxidation of pea lipids in the presence of various matrices. Conditions underlying the results depicted in sections a and b of Gigure 30 are exactly identical, except for the parameter of light (absent in a, present in b). The time coordinates in Figure 30 are drawn to the same scale. The most obvious difference between the two sets of data is the ratio of the ordinate scales: 8 $\mu l \cdot mm^{-1}$ for data obtained from experiments conducted in the dark (Figure 30a) and 400 $\mu l \cdot mm^{-1}$ for data obtained from experiments conducted in light (Figure 30b), or a ratio of 1 to 50. Since graphs in a are about twice as high as those in b, the real ratio of magnitudes (dark to light) is about 1 to 25. That is, under the intense lightling employed, consumption of oxygen (as volume of ambient air) was about 25 times as great as that which occurred when light was excluded.

In all experiments conducted in the dark (Figure 30a) the maximum oxygen uptake was achieved by NL (neutral lipids) in the absence of β-carotene. Indeed, oxidation of NL outstripped oxidation of NL + β-carotene in all

Figure 30. Volume changes in dry Gilson respirometer flasks, containing four pea matrix discs impregnated with pea lipids ± β-carotene, and maintained at constant pressure for 50 h. Plotted points are based on duplicate values; temperature, 50°C. Positive values indicate volume decrease (apparent O_2 uptake); negative values indicate volume increase (net gaseous evolution).

a. In the dark; 15 points plotted.

b. In intense light; 25 points plotted.

Abbreviations:

Alb = pea albumins

Glo = pea globulins

Cel = cellulose (filter paper)

Amyl = amylose

A-P = amylopectin

Pect = pectin

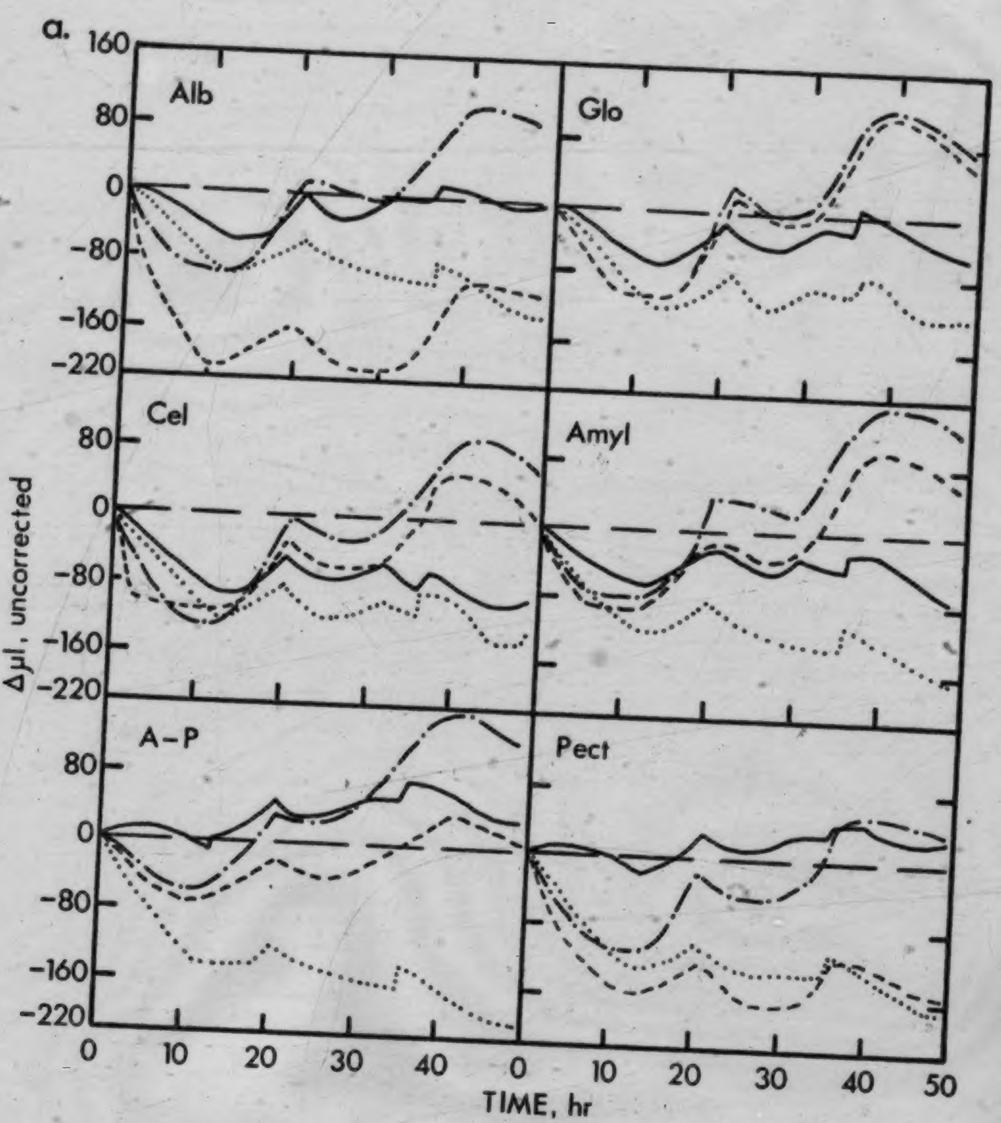
Legend: Matrix impregnated with

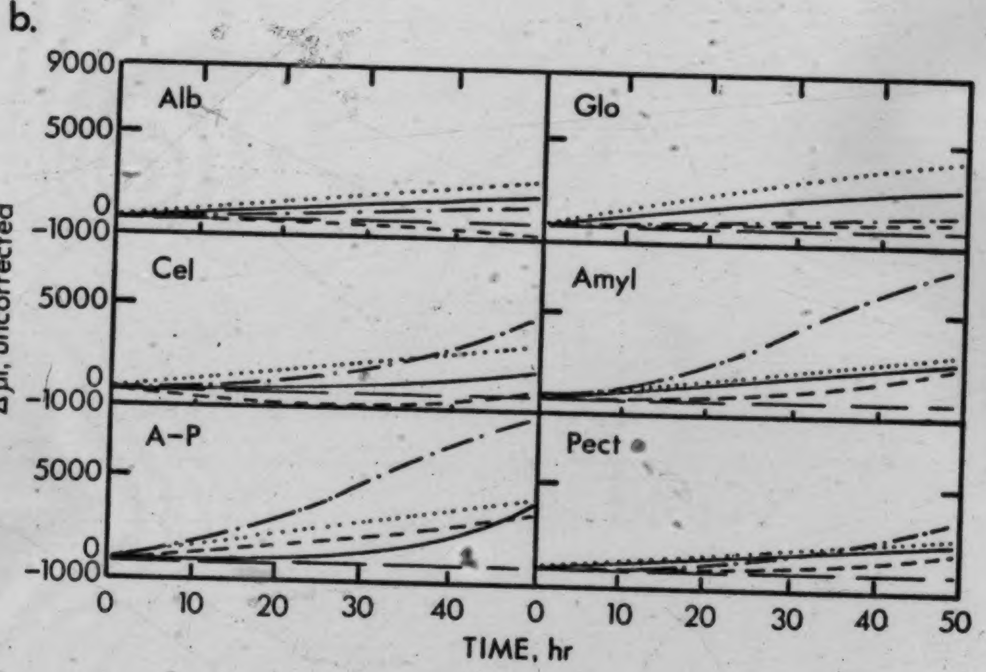
.... pea polar lipids

———————— pea polar lipids + β-carotene

—.—.—. pea neutral lipids

------------ pea neutral lipids + β-carotene


a.
160
80
0
-80
-160
-220
Alb
Glo
Cel
Amyl
A–P
Pect
Δμl, uncorrected
0
10
20
30
40
0
10
20
30
40
50
TIME, hr


b.
Alb
Glo
Cel
Amyl
A–P
Pect
9000
5000
0
–1000
5000
0
–1000
5000
0
–1000
Δμl, uncorrected
0
10
20
30
40
0
10
20
30
40
50
TIME, hr

light experiments, as well (Figure 30b). However, in the dark experiments, one is thrown back on much speculation, since more of the plotted points are below zero than above, which indicates a net migration of matter from the solid phase to the gaseous. Among matrices impregnated with PL (polar lipids), only two curves rose substantially above zero (net volume decrease in flasks). These were PL + β-carotene associated with amylopectin and pectin. A certain uniformity in the appearance of peaks is discernible in all curves obtained from the experiments conducted in the dark (i.e., broad peaks centered at about 20 h and 40 h). Thus, the experiments conducted in the dark were generally characterized by a net expansion of gas phase in the first 12 to 15 h, followed by contraction up to about 20 h, after which different patterns set in. These latter were a levelling off, rising or falling, depending on the nature of the lipids and matrices involved.

A pattern common to all six matrix categories tested in the dark emerges: the presence of β-carotene retarded oxidation of NL (antioxidant) and stimulated oxidation of PL (pro-oxidant).

In the light (Figure 30b) NL were uniformly protected by β-carotene, also (as in Figure 30a), but, in contrast to the dark experimental results, so were PL protected from oxidation by β-carotene.

Although impregnating solutions were made up to conform to a NL/PL ratio of 1.8 (see Methods B.2.e.), actual weighing of discs after freeze-drying indicated that the ratios obtained were 1.89±3.40%, averaging three discs for each category (NL: 2.16 mg · cm disc surface^{-2}; PL: 1.14 mg · cm disc surface^{-2}).

Figure 31 is a compilation of graphs representing oxygen uptake per matrix disc in sealed flasks throughout 50 h-runs. The plotted points are based on calculations made from periodical pO_2 readings taken in an atmosphere (originally of ambient air) of known volume, under known pressure at 50°C. Although the ordinate is labelled oxygen uptake per disc (μM), true uptake is only indicated by a curve of positive slope. In all graphs, with the exception of that for pectin + PL + β-carotene (if minor dips are disregarded), there are negative slopes, which indicate increasing headspace oxygen. The highest values of oxygen uptake were registered in the dark, using globulins + PL + β-carotene; the lowest uptake was registered with the same matrix category in the light.

In Table 12, which is based on data presented in Figures 30 and 31, can be found relative maximum oxygen uptake displayed by NL and PL associated with various matrices, with and without β-carotene. The arrangement is in decreasing order of susceptibility to oxidation, from left to right.

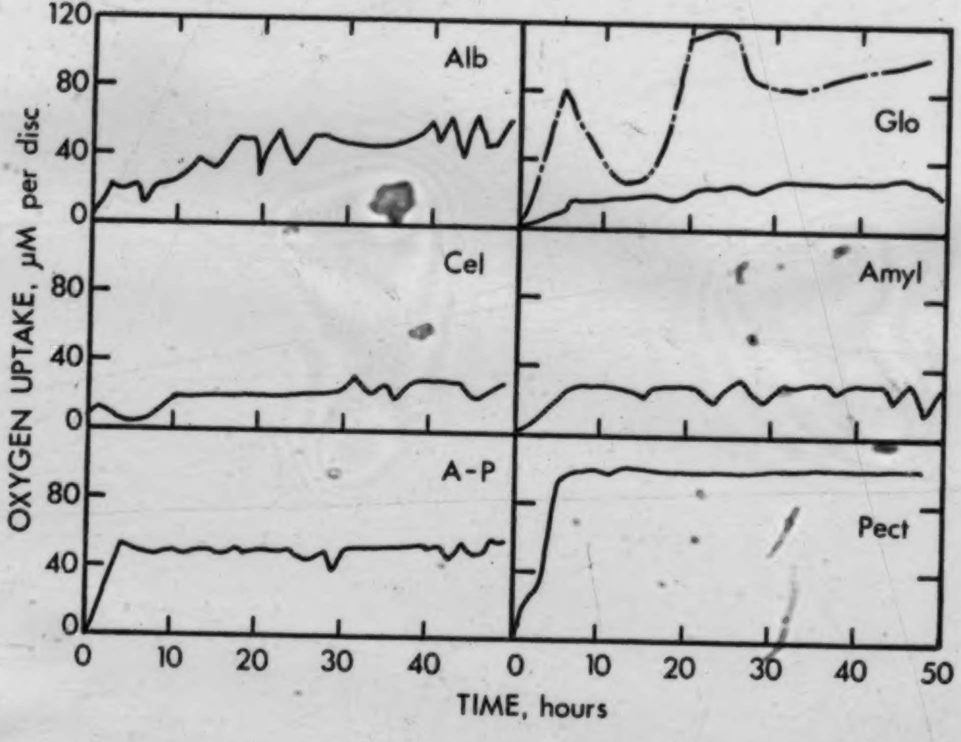


Figure 31. Oxygen uptake, recorded polarographically, per matrix disc impregnated with pea polar lipids + β-carotene, over 50 h at 50°C. Abbreviations as in Figure 30. Plotted points based on single values. Number of points ranged from 31 to 45 per run. Legend: one experiment in the dark (-·-·-); the others in intense light (——).

Table 12. Relative Maximum Oxygen Uptake Displayed by Pea Neutral Lipids (NL) and Polar Lipids (PL) Associated with Various Matrices, with and without Beta-Carotene, During 50 Hours at 50°C.*

Instrumental Method	Lipid Catagory	In the Dark	In the Light
Gilson respirometer	NL	A-P > Amyl > Glo > Alb > Cel > Pect	A-P > Amyl > Cel > Pect > Glo > Alb
	NL + β-carotene	Glo > Amyl > Cel > A-P > Alb > Pect	A-P > Amyl > Pect > Glo > Cel > Alb
	PL	Glo > Alb > Cel > Amyl > Pect > A-P	A-P > Glo > Amyl > Cel > Alb > Pect
	PL + β-carotene	A-P > Pect > Alb > Glo > Amyl > Cel	A-P > Amyl > Glo > Pect > Alb > Cel
Beckman Lab, O_2 Analyzer	PL + β-carotene	Glo > Pect > Alb > A-P > Amyl > Cel > Glo	

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* Based on data presented in Figures 30 and 31.

V. DISCUSSION AND CONCLUSION

A. Concerning Lipoxygenase.

In the hands of Eriksson and Svensson (1970) pea lipoxygenase was obtained as an homogeneous protein upon ultracentrifugation. Procedure I of the present investigation was taken directly from their paper. Instead of testing the effect of cyanide on lipoxygenase, they monitored hemoprotein by assaying specifically for peroxidase and catalase activities and by analyzing pyridine ferrohemochrome absorption bands in the U.V. and visible regions of the spectrum. After Sephadex G 150 chromatography, they had separated most of the lipoxygenase from peroxidase and catalase. After the first DEAE-cellulose chromatography step, utilizing the steep salt gradient, only minor peroxidase and catalase activities were left in the concentrated lipoxygenase fraction. Rechromatography on DEAE-cellulose with a less steep salt gradient freed lipoxygenase completely from the other two enzymes. By assuming a partial specific volume of 0.75 ($ml \cdot g^{-1}$) they calculated a preliminary molecular weight of 75,000. Partial specific volumes of 0.74 and 0.72 were obtained with different methods of calculation from amino acid analysis data, yielding calculated molecular weights of 71,845 and 67,000, respectively. In any case, the values are open to question because methionine and tryptophan residue values were lacking.

The two isoenzymes from ripe green peas, which were characterized by Weber, Arens and Grosch (1973) as being identical to so-called carotene oxidase and as corresponding to lipoxygenases-2 and -3 of soybean described by Christopher et al. (1970, 1972), were doubtless encountered in the present study. Using DEAE-cellulose column chromatography, Weber, Arens and Grosch (1973) separated the pea enzyme into two peaks, each with an optimum activity at pH 6.5. They reported that the elution pattern corresponded to lipoxygenase-2 and -3. Further, they reported a molecular weight of 78,000 for one of the pea isoenzymes (details and data not reported, however). The SDS gel molecular weight of 74,000 previously reported from this laboratory (Haydar and Hadziyev, 1973a), probably corresponds to the band representing 76,000 reported here (Figures 17 and 18). The inference that this lipoxygenase isoenzyme from the pea, which resisted storage (cf. Figure 20), corresponds to the soybean isoenzyme-3 of Christopher et al. (1972) is supported by evidence of the kind reported by the latter. The striking nonlinearity of activity as a function of enzyme concentration (Figure 10) mimics very closely that reported for soybean isoenzyme-3, as does, also, the response to calcium depicted in Figure 11 (see Christopher et al., 1972, p.61).

Specific activities in Table 2 are not accompanied by turnover numbers, because it is not likely that the enzyme preparations in each case represent a single, pure isoenzyme of pea lipoxygenase (see below, concerning cyanide inhibition). If one were to assume this and that the bands observed on SDS gels represented components of the parent protein molecule of molecular weight 106,000, then "turnover numbers" of 486, 3820 and 1410 for isoenzyme-3 of pea lipoxygenase could be calculated when Procedures I to III were employed, respectively. But turnover numbers are not justified unless one has at least several lines of evidence indicating that the enzyme in question is homogeneous, if not indeed crystallized. The relatively low activity of the Procedure I preparation as compared to that prepared by Procedure II can be attributed to calcium inhibition. The intermediate value of the Procedure III preparation reflects reduced purity resulting from the omission of precipitation with ammonium sulfate.

The effect of aging in cold storage (Figure 12) indicates that this enzyme is generally rather stable after purification, as it is in the natural environment of the intact tissue of the pea, but that, immediately after purification (within 24 h), a sudden loss of some activity occurs. This latter may pertain to irreversible conformational changes which introduce some steric hindrance near active sites.

It is well documented that cyanide has no effect on pure lipoxygenase from soybean (see *e.g.*, Pistorius and Axelrod, 1974). Until lipoxygenase from peas is likewise readily crystallizable and available for extensive studies, which is theoretically possible, one can only conjecture that it, too, is cyanide insensitive. Thus, one assumes that any inhibition is a result of co-precipitation and/or co-chromatography of some other protein catalyst which is cyanide sensitive (such as heme protein). From Table 3, one sees that this is the case here. Peak 3 of Procedure I, which is inhibited 96% by calcium, is also strongly inhibited by cyanide (87%). The latter inhibition points to contamination by heme protein, but probably there is substantial isoenzyme-3 (adopting the soybean lipoxygenase terminology of Christopher *et al.*) as suggested by calcium inhibition. Slight procedural differences (as seen for instance between Procedures I, II and III) could cause subtle shifting of elution orders of isoenzymes. Ample testing of elution peaks with cyanide and calcium would be required before a more precise statement of conditions could be made about optimal conditions for molecular sieving and ion exchange chromatography of a given pea lipoxygenase isoenzyme. Full purification may never be achieved by this method alone, but the use of other techniques, such as preparative electrophoresis, was beyond the scope of this thesis.

Procedure III, which was that used by Weber, Laskawy and Grosch (1973), is reported by them to yield pea lipoxygenase isoenzyme-3 in peak 1 and isoenzyme-2 in peak 2. However, as the results in Table 3 indicate, it is not possible to state categorically on the one hand that the first two peaks represent carotene oxidase and, on the other, that carotene oxidase is nothing more than lipoxygenase (isoenzyme-2 and -3, in the pea), because cyanide insensitivity is generally established for lipoxygenase. Further, the lack of homogeneity is evidenced by peak shoulders (Figure 13 of this report and Weber, Laskawy and Grosch, 1973, p.327).

That the higher iron salt concentrations (Table 4) had a pronounced and instantaneous pro-oxidative effect on linoleic acid before the addition of enzyme preparation was to be expected, since iron is one of the best known catalysts of fat autoxidation. The concentrations were high enough to eliminate the induction period. That the lower iron salt concentrations stimulated biocatalytic activity may have been additive. The induction period, as evidenced by no oxygen uptake before addition of enzyme preparation, was eliminated by the lower activation energy required by the latter. Not so facile is the interpretation of the observation that the pro-oxidative effect of 50 mM Fe^{2+} on linoleic acid was inhibited by addition of enzyme, whereas addition of enzyme to autoxidizing linoleic acid in the presence of 50 mM Fe^{3+} had no effect. If the iron atom in the enzyme is necessarily in the ferric state for activity, as the evidence presented by Pistorius and Axelrod (1974) strongly suggests, addition of a relatively large amount of Fe^{2+} (50 mM) would, by simple mass action, reduce some Fe^{3+} of the enzyme molecules to Fe^{2+}, the inactive form. By the same argument, addition of enzyme to linoleic acid autoxidizing in the presence of 50 mM Fe^{3+} would not change the oxidation state of lipoxygenase iron. The inhibition of enzymic oxidation of linoleic acid observed in the presence of the 1000-fold higher iron salt concentrations has yet to be explained. Sufficient autoxidation was taking place to render some substrate unavailable for enzymic attack by converting it to hydroperoxide. The remainder was not so rapidly oxidized as in the absence of iron, because the enormous metal ion population, by some sort of interaction with enzyme protein, was clogging the delicate machinery. This speculation seems reasonable in view of Tappel's mechanism, which postulates multiple active sites (Tappel, 1963). Although copper is known to poison many plants and animals, no proposed mode of action in the lipoxygenase system will be attempted here.

Haydar et al. (1975) reported that Homesteader pea seeds kept in cold storage for more than six months retained activity of only one lipoxygenase enzyme. After extraction and purification by ammonium sulfate fractionation, gel filtration on Sephadex G 150 and DEAE-cellulose column chromatography, the enzyme, which had the distinct elution profile and inverse dependence of activity upon enzyme concentration reported here (Figures 4, 5 and 10, this report), showed the same pH and substrate dependence as depicted in Figure 14. In fact, it was the same enzyme and the data, including calcium inhibition, suggest that it corresponds to the recently characterized soybean lipoxygenase-3 of Christopher _et_ _al_. (1972). These authors reported pH 7.0 as the optimum for activity when linoleic acid was used as substrate for isoenzyme-3. They did not test the enzyme with other substrates, although earlier they had reported that the soybean lipoxygenase-1 and -2 corresponded roughly with the classification "fatty acid (or linoleic acid) lipoxidase" and "triglyceride (or trilinolein) lipoxidase", respectively (Christopher _et_ _al_., 1970). Each of these purified isoenzymes was active on both substrates, relative activities being a function of pH. The preference for trilinolein of the enzyme reported here tends to rule out correspondence with isoenzyme-1 and suggests a peculiar architecture of the isoenzyme for handling the triglyceride skeleton most efficiently. The drop in activity as pH increases from the optimal pH 7.0 is reversed after pH 8.5, suggesting that a solubility effect on the larger triglyceride molecule was operative, which did not enhance reaction with the simpler substrate molecules (see Figure 14).

Christopher _et al_. (1970) reported, moreover, that difference in heat stabilities differentiate the two soybean lipoxygenases, isoenzyme-1 (Theorell) and isoenzyme-2. In their hands the former had a half-time of survival of 25 min at 69°C. while the latter was at least 36 times less stable. Comparison with results in Table 6 would probably rule out correspondence of the tested pea lipoxygenase isoenzyme with soybean isoenzyme-1, since over half of the activity was lost in only 20 min at 60°. Certainly isoenzyme-2 could be excluded on heat stability grounds, since the enzyme reported here was more stable than the soybean isoenzyme-1, not less.

The conflicting statements to be found in the literature concerning the phenomena associated with enzymic carotene bleaching could be resolved if researchers could concern themselves with obtaining pure enzyme preparations rather than with polemics. Although soybean Theorell enzyme (isoenzyme-1 of Christopher _et al_.) is a poor carotene bleacher in the presence of linoleic acid when pure, commercial preparations contain considerable peroxidase activity (Eriksson and Svensson, 1970, p.450). This is substantiated in Figure 21.

The more thorough investigations into the identity of carotene oxidase, such as the paper by Kies _et al_.

(1969), take purity into account, as well as heat stability. In reading the report of Weber, Arens and Grosch (1973), one is struck by the lack of scepticism toward their own conclusions. They claim to refute Kies et al., who impute carotene bleaching activity to some heat sensitive factor, without any heat testing. As the present report has shown, ample carotene bleaching can be obtained with pea lipoxygenase prepared by column chromatography, etc., but certainly no claim can be made to a pure enzyme preparation. In Table 13, are set out some data for comparison. Weber, Arens and Grosch take the view that, by their method of preparation, the nearly equal values for Q (quotient = ratio of lipoxygenase activity to that of carotene oxidase) obtained with raw extract and purified enzyme is sufficient to prove the identity of the latter with lipoxygenase alone. On the contrary, this merely underscores that the purification procedure yields a biocatalytic preparation of protein nature capable of catalyzing coupled oxidation of linoleic acid and β-carotene.

The difference in turnover values presented in Table 13 (reaction mixture c is according to Ben Aziz et al., 1971) for β-carotene from the 30th to the 90th sec can be attributed to:

1. Assumption of a molecular weight of 106,000 in this report for the enzyme preparation

Table 13. Turnover Data Derived From Beta-Carotene Bleaching Systems. A Comparison of Results of Different Workers

Enzyme Preparation	According to										
	Weber, Arens and Grosch (1973)				This report						
	30th to 90th sec					30th to 90th sec		First 15 sec			
	Turnover*		Q**	*** Turnover number	Reaction mixture	Turnover	Turnover				
	Linoleic acid	β-carotene				β-carotene	number	Linoleic acid	β-carotene	Q	Turnover number
Raw pea extract	9.5	2.5	3.8								
Pea lipoxygenase isoenzyme	5.9	1.5	3.9	~10^3							
Pea lipoxygenase (Procedure I, first DEAE-cellulose column effluent peak)					a	1.06	11.1	5.0	1.09	4.6	11.6
					b	1.96	20.8	4.2	1.16	3.6	12.3
					c	1.59	16.8	4.2	5.15	0.8	54.6
					d	2.05	21.6	5.2	0.51	10.2	5.4
					e	2.46	26.2	5.0	0.62	8.1	6.5
Soybean 1:poxygenase					a	0.32	3.4	107	1.75	61.1	18.6
					b	1.81	19.3	92.8	2.18	42.6	23.3
					c	4.98	53.2	23.6	4.53	5.2	48.3
					d	5.02	53.5	43.0	0.45	95.6	4.8
					e	0.47	5.0	18.1	0.13	139	1.4

* $\underline{\mu M}.10^{-3}.ml^{-1}$ ** Quotient = $\underline{\mu M}$ linoleic acid. $\underline{\mu M}$ β-carotene^{-1}

*** Molecules of β-carotene.mole enzyme $^{-1}$.min^{-1}

represented by the first DEAE-cellulose column effluent peak, Procedure I. Weber, Arens and Grosch report a value of 78,000 but do not indicate their method of determination or clarify whether they identify it with isoenzyme-2 or -3.

2. Modification by Weber, Arens and Grosch of the substrate of Ben Aziz et al. as follows: final concentration of linoleic acid, reduced to one half; final concentration of β-carotene, reduced to 0.679 or about two-thirds.

3. Measurement at pH 6.5 by Weber, Arens and Grosch as compared to pH 7.0 for the assays in this report.

4. Unknown experimental parameters, including the use of ripe green peas, var. Mignon, by Weber, Arens and Grosch as opposed to dry stored pea seeds, var. Homesteader, here.

Nevertheless, the purpose of each of the two studies was different. Weber, Arens and Grosch were attempting to demonstrate the identity of lipoxygenase (isoenzyme-2 and -3) with carotene oxidase and the present study was designed to elucidate the parameters influencing β-carotene destruction. To summarize, it can be said that the time factor is important, as is indicated by comparison of results obtained in the first 15 sec with those obtained from the 30th to the 90th sec (Table 13) and by the shapes of the curves in Figures 22 and 23. Also, the ratio of β-carotene concentration to that of lipid substrate determines to a large extent the amount of enzymic coupled oxidation of β-carotene and unsaturated lipid. Although it was not tested, it is possible that these parameters are likewise important in any non-enzymic oxidations involving carotene and unsaturated lipids.

B. Non-enzymic Oxidations.

The first set of preliminary experiments with non-enzymic oxidation of linoleic acid in the presence of β-carotene and crocin (Figure 27, dry systems) indicated that this purified neutral lipid was protected from oxidation by crocin about equally at the three concentrations employed, whereas in the presence of β-carotene, it was only protected at the lowest concentration. At the higher concentrations, β-carotene was pro-oxidative. But in solution, both pigments were pro-oxidative at the higher concentrations (Figure 28, non-enzymic systems). The latter results are limited only to the first few seconds and should not weigh too heavily. The conflicting data only provoked further work. Although no mechanism is evident, it is possible to conclude that β-carotene in the dark can be pro-oxidative at room temperature in enzymic and non-enzymic systems and appears to be anti-oxidative in enzymic oxidations only in unnaturally high concentrations, which agrees with results obtained in the β-carotene destruction experiments. This is also consistent with the observation reported by Haydar (1974), who examined the role of β-carotene during photoxidation of butter fat, that β-carotene in low concentration seemed to be pro-oxidative in the dark. Before further inferences could be made, a repetition of these experiments in the presence of light recommends itself.

Concerning the preliminary work with wheat proteins, one could interpret the negative values in Figure 29b (40°C.) as representing products of cellulose autoxidation which are well volatilized at 40°C., but not at 25°C. These products do not react with oxygen unless heated to 50°C., at which temperature the tendency for gas phase expansion is nullified by oxygen uptake. A monolayer of adsorbed atmospheric water molecules is ruled out beyond reasonable doubt by the fact that it did not occur appreciably at 50°C. Freeze-drying technique and desiccator storage regimens were uniformly executed and each matrix category was handled as one batch, thus cutting across temperature categories. Furthermore, monolayer evolution persists only for a few hours at the most, not for 50 h.

Data resulting from the more vigorous oxidations at 50°C. were less complicated, probably because the intricacies manifest in a and b of Figure 29 were negligible compared to oxygen uptake. In explanation of the result that matrix + linoleic acid was lowest in oxygen uptake (at 50°C.) when the matrix was globulins (Table 11), it is proposed that, since linoleic acid is a slightly polar molecule, it is probably held more tenaceously by globulins than by the other matrices due to the more highly charged surface presented by globulins. If this circumstance hindered oxidative reaction, then the speculation would seem reasonable. It is at best a working hypothesis to be tested.

When comparing Figure 29c (Park) with Figure 29d (Thatcher) results, the greatest difference between oxygen uptake of matrix + linoleic acid is found to be where matrix consisted of globulins. Skura (1972; pp. 55-60, 62-74) has shown with urea and SDS gels that differences in distribution and relative quantities of protein components in glutenins, globulins and gliadins of Park and Thatcher varieties of wheat do exist. Partial substantiation of the findings presented here is found in Skura's work, inasmuch as qualitative differences (molecular weights of major bands in the 29,000 to 67,000 range) are greatest between the globulins of these two varieties.

Oxygen uptake, in Figure 29c and d, of matrices combined with linoleic acid and β-carotene is greater than that of the same matrices combined with linoleic acid alone, when the matrix in question offers a charged or hydrophilic surface (cellulose, globulins, glutenins*). On the other hand, when the matrix is composed of gliadins,* the opposite result ensues: oxygen uptake is greater when gliadins are combined with linoleic acid alone than when β-carotene is included.

Since the reactions occurred in the dark, we can assume that β-carotene functioned in the autoxidation only as a conjugated unsaturated hydrocarbon and not, also, as a light capturing pigment. One can imagine a greater hydrophobic affinity of β-carotene for the gliadins than for the other matrices. Assuming autoxidation of linoleic acid to be enhanced by partial matrix binding (by a more stationary orientation in space and, also, by suppression of fatty acid dimer formation), then β-carotene could function antioxidatively by "competitive inhibition" (greater affinity than linoleic acid for the surface of gliadins).

The experimentally established pro-oxidative effect could be explained on the basis of the ample π electrons of β-carotene (11 double bonds). They could induce autoxidative chain initiation in the linoleic acid molecule with which they are in intimate contact by causing electron shifts which favor methylene hydrogen abstraction. This postulated mechanism would be hindered or nullified by the gliadins matrix only, in whose presence the π electrons of β-carotene are no longer as free. This interpretation could be tested by further experiments in which crocin, e.g., was used instead of β-carotene. Such investigations should include a wide range of carotenoid concentrations.

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* Glutenins are not characterized by charged groups in the way that globulins are, but have many exposed disulfide linkages, whose localized electron densities are sufficient to put them into this group. Gliadins have practically no charged amino acid residues and contain mainly single polypeptide chains; the disulfide bridges are substantially buried (Wall et al., 1972, p.120).

An interesting task for further investigation would be to establish the extent to which free sulfhydryl groups effect the oxidation behavior in Gilson experiments (both in light and dark) as described here, using proteins prepared in the usual way and, also, prepared in denaturing solvents (such as urea), which would expose buried sulfhydryls. This proposed work would compare oxidations with and without β-carotene, as well, with the idea of elucidating its mode of action.

When attempting to interpret the results of the experimental non-enzymic oxidations of pea lipids in the presence of different matrices, one is confronted first of all by the gross difference between oxygen uptake occurring in the dark and that occurring in the presence of light. As to whether these processes occur by different mechanisms, it can only be pointed out that leading scientists in the field do not agree. The view held by Scott (1965, p.95 ff.), that photoxidation occurs by the same chain propating steps as autoxidation taking place in the dark, is opposed by that of Lundberg (1962, p.41 ff.), who holds that the mechanisms are basically different, especially in the presence of a photosensitizing pigment. Perhaps, in photoxidations of the sort reported here, autoxidation occurs simultaneously in spite of the fact that it cannot be demonstrated to be subject to normal antioxidant action (Lundberg's main argument). This problem is intimately involved with the question of oxidation rate. The present results offer only the clue that they appear to represent different mechanisms in the dark as compared to the light reactions, because the dark curves display a roving course, and those obtained in the light are rather uncomplicated, approaching steadily rising straight lines. But this interpretation overlooks the question of rate. The reactions depicted in Figure 30a did not reach the linear stage (steady state of oxidation equivalent to maximum rate) as did those depicted in Figure 30b, and the question of mechanism remains unsettled.

No fewer than 12 of the 24 curves resulting from the pea matrix experiments in the dark persisted in the negative zone for 50 h. The Gilson respirometer registers only net gaseous content in flasks. Doubtless oxygen was consumed, but the products of reaction must have been incompletely oxidized, substantially gaseous at 50°C. and complex in the extreme, since each curve had its own profile. By contrast, experiments conducted in the light led to more complete oxidation, because the Gilson readings were predominantly positive (net shrinkage of headspace gas phase). The oxygen uptake values, being of a greater order of magnitude, were sufficiently large to nullify any minor gas phase expansions of the kind recorded in the dark. An interesting difference in results from pea matrix experiments in the dark as compared to wheat matrix experiments in the dark, which shows that pea NL as a group did not respond as did purified linoleic acid, is that oxygen uptake of matrices impregnated with NL + β-carotene was always less than that of the same matrices impregnated with NL alone, when the matrix offered a charged or hydrophilic surface. One must design experiments more simply, with each lipid precisely defined, before a well-founded mechanism could be deduced.

The differences apparent among the matrix categories are difficult to interpret. Probably the relationship summarized under "In the light" in Table 12 are more meaningful than those under "In the dark", because the latter suffer from the complicating superimposed undulations (these could have originated in the filter paper itself, since that parameter was common to all categories).

Table 14 focuses attention on the results obtained in the light, therefore, and compares those of Haydar and Hadziyev (1973b) with results obtained in this study (from Table 12, Gilson). The two sets of data show agreement with regard to relative rates of oxygen uptake by NL and PL associated with pea proteins, but not when these lipid categories were associated with the various carbohydrates. This comparison omits β-carotene from consideration.

A critique of the experimental autoxidation of neutral lipids in the dark (including preliminary experiments) is not easy because, although β-carotene has been shown to be pro-oxidative in the presence of linoleic acid when combined with cellulose, wheat globulins and glutenins, it is distinctly anti-oxidative in the presence of linoleic acid combined with gliadins. Where pea neutral lipids were present as a mixture, they were uniformly protected by β-carotene in the dark and in the light, regardless of matrix. Polar pea lipids were not protected from autoxidation by β-carotene in the dark, but were oxidized faster in its presence, regardless of matrix. Only in the light was β-carotene antioxidative for polar pea lipids in all systems tested. From this evidence it can be concluded, that, although selection of a particular matrix influences the amount of non-enzymic oxidation of pea PL or NL significantly, β-carotene effects the oxidation also. In the light, the pigment is

Table 14. Relative Maximum Oxygen Uptake by Pea Neutral Lipids (NL) and Polar Lipids (PL) at 50°C. In the Light. A Comparison of Results of Different Workers*.

According to

	Haydar and Hadziyev, 1973b, pp. 776-77.	Table 12, this report (Gilson respirometer)
NL	Cel > Amyl > Pect > A-P	A-P > Amyl > Cel > Pect
	Cel > Glo > Alb	Cel > Glo > Alb
PL	Pect > A-P > Amyl > Cel	A-P > Amyl > Cel > Pect
	Glo > Cel > Alb	Glo > Cel > Alb
NL/PL	1.66	1.89
Measurement Period	20 to 45 h	50 h

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* Abbreviations as for Figure 30.

antioxidative for both lipid categories. In the dark, it is antioxidative for NL and pro-oxidative for PL.

Further research, which could link up the conclusions discussed above (concerning the role of wheat proteins in autoxidative processes) with results of the pea lipid oxidation work, would include some experiments with pea lipids as here described and gliadins as a matrix. The purpose would be to determine whether the rôle of β-carotene would be reversed, because at present there are not enough facts available. Conversely, similar data based on a polar lipid, such as phosphatidyl choline, for example, are lacking.

To conclude the discussion, a word about the headspace oxygen analyzer (Figure 31) is in order. These results are potentially far more valuable than those derived from the Gilson, because they are in terms of oxygen rather than mere volume changes. Besides the obvious need for multiple results and an unequivocal ruling out of any leaks (which could explain an apparent reversal of oxygen uptake by the discs), the want of substantial data is conspicuous. In other words, can the bottom two horizontal columns in Table 12 be reconciled? Such data, both for matrices with and without β-carotene, would have the highest priority in further investigation of the role of β-carotene.

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APPENDIX

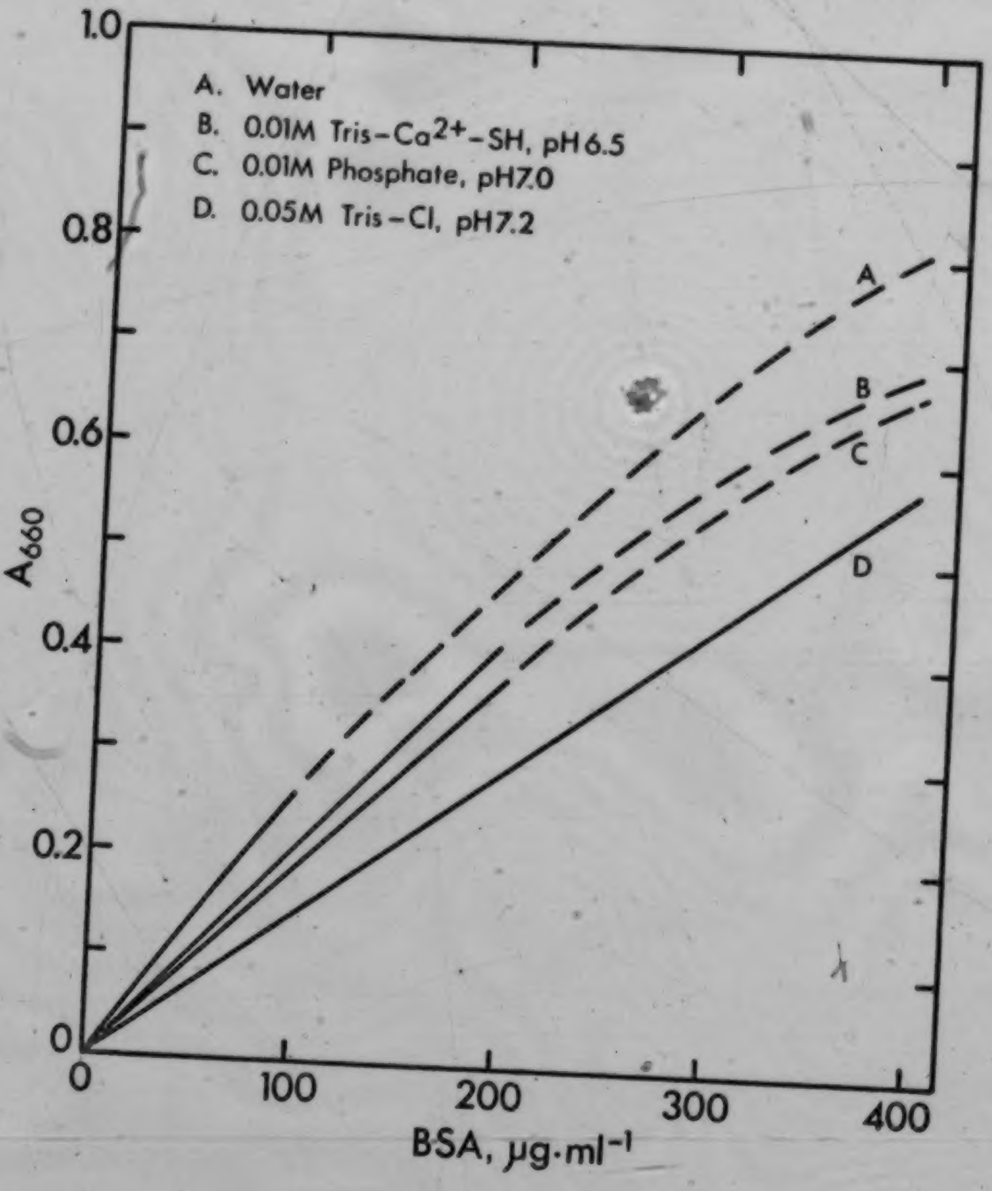

Figure A1. Lowry protein calibration curve according to Oyama and Eagle (1956). Departure from linearity indicated by (----).

Derivation of Formula for Converting Oxygraph Readings to Absolute Values

The polarographic oxygen probe measures oxygen pressure change in the reaction medium as a function of time. In order to convert to $\mu\underline{M}\ O_2 \cdot \text{min}^{-1}$, the following factors must be considered:

1. O_2 depletion $\cdot\ \text{min}^{-1}$ as a fraction of 100% (or unity) saturation of the medium with atmospheric O_2. Let this be X, the experimentally determined value from the Biological Oxygen Monitor (oxygraph).

2. Mole fraction of air represented by oxygen (0.2095).

3. Prevailing barometric pressure (mm Hg).

4. Volume of medium (ml).

5. Nature of medium. If aqueous, the ml of O_2 dissolved per ml medium is assumed to be the same as for water; i.e., the Bunsen coefficient (α) is employed (cf. Figure A2).

6. Temperature of medium (°K.).

7. Volume of one mole of O_2 at S.T.P. (22.414 liters), or, 1.0 $\mu\underline{M}\ O_2$ = 22.414 μl of O_2 at S.T.P.

Thus,

$$1\ \mu l\ O_2 = \left[\frac{\left(\frac{273}{°K.}\right)\left(\frac{\text{mm Hg}}{760}\right)}{22.414}\right]\ \mu\underline{M}\ O_2$$

Assuming the reaction cell to contain Y ml (final

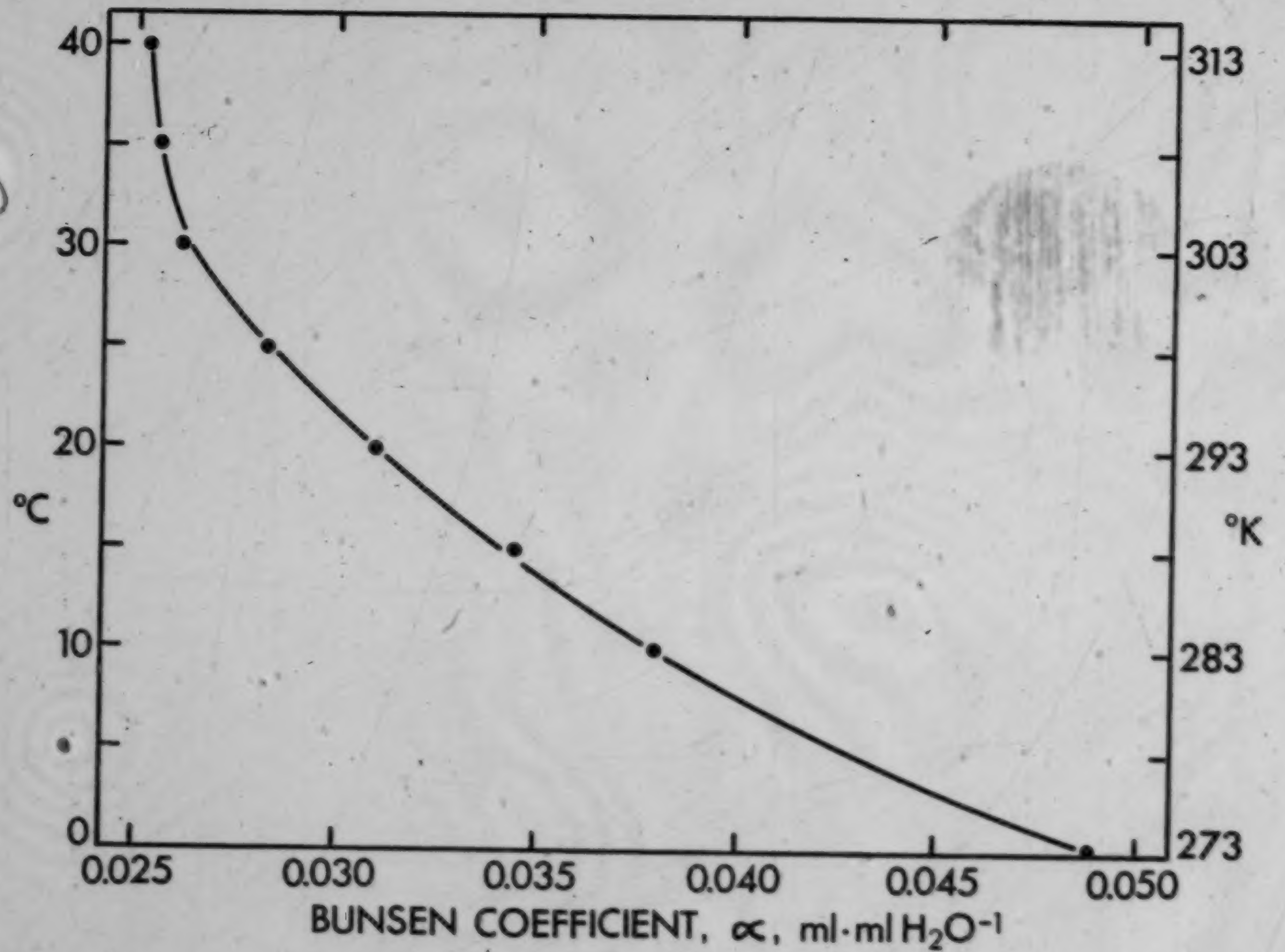

Figure A2. Solubility of O_2 in water at one atmosphere. Data from Washburn, 1928, pp. 257, 271.

volume), the conversion formula becomes

$$\mu\underline{M}\ O_2 \cdot Y\ ml^{-1} \cdot min^{-1} =$$

$$(X)(Y)(0.2095)(\alpha)\left[\frac{mm\ Hg}{760}\right]\ \left[\frac{1000}{22.414}\right]\ \left[\frac{273}{°K.}\right]\ \left[\frac{mm\ Hg}{760}\right]$$

When Y = 3.0 ml, as was the case throughout these studies, and °K.=298 (25°C.), as was most common, the formula becomes

$$\mu\underline{M}\ O_2 \cdot min^{-1} = (X)\ (mm\ Hg)^2\ (1.2459)\ (10^{-6}).$$

Evaluation of Calibration Curve (Figure 17) Used in Determination of Molecular Weights

The best straight line, based on the six points (x, y) established by the purified protein standards,

Rm (x)	Mol. wt. (y)
0.14	160,000
0.68	67,000
0.91	45,000
1.00	36,000
1.19	25,000
1.62	12,400

was evaluated by the University of Alberta Computing Services by the method of least squares (ON AT 09:58.20, OFF AT 10:06.48, Wed., Oct. 2/74). The best values of m(slope) and b(intercept of y-axis) were found to be

$$- 0.16846$$

and

$$203,230 ,$$

respectively. The 95% confidence interval for the slope was found to be

$$- 0.18471 < m < - 0.15221.$$

Thus, the line used for molecular weight determinations, where the slope = - 0.1837, the negative antilog of - 0.73588 (cf. Figure 17), falls within this confidence interval.

Note Concerning Assessment of Precision of the Gilson Respirometer

The relative standard deviation is a measure of agreement among repeat determinations which expresses precision (%), in terms of the quantity being determined. It is calculated by dividing the standard deviation, S, by the mean value, $\bar{x}$, of n determinations of individual x_i values. S is obtained by

$$S = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n - 1}}$$

and relative standard deviation as percent is

$$(S / \bar{x}) \times 100$$

The manufacturer's instructions for testing the instrument for leaks were modified insofar as the second temperature equilibrium was only 0.3°C. higher than the first, rather than 1.0°C. This was done for the same reason that frequent manometer adjustments were necessary to obtain even approximate reproducibility. It was discovered early, namely, that conditions (such as long intervals between adjustments, very rapid oxidations and leak testing regimens with temperature changes greater than 0.3°C.) which cause manometer fluid meniaci to travel beyond the right angle bend of the manometer tubes, introduce enormous errors. The interpretation is probably that additional friction is introduced thereby and that some leakage in tygon tubing occurs. This problem does not exist with the more expensive all-glass models (personal communication from Mr. Hugh Kay, technician to Dr. N. Colotelo, Plant Science Department, University of Alberta). The remedy is clear, but expensive in terms of labor.

Below is given detailed description of a typical test carried out on the GR 20 in order to dispel, by objective observation, any polemics relating to this instrument.

The volumes of 20 manometer flasks, nominally of 15 ml capacity, were determined to the nearest 0.1 ml with serological pipets and distilled water. They were then emptied and oven dried. All equipment and water were equilibrated with ambient room temperature (22.5°C.). The empty, dry flasks, balanced by an empty, dry reference flask (250 ml), were sealed to manometer arms as usual and allowed to equilibrate with all values open. The menisci were set to index lines and all microvolumeters were set at 300 µl. The operator valves were closed. After 30 min, the water bath still at 22.5°C., the menisci were observed to be at the index settings as expected. The temperature was then brought gradually to 22.8°C., manometer adjustments being made continually. 30 min at the new temperature were maintained without need for further adjustment. The procedure was terminated and readings recorded (cf. Table A1).

$$\text{Variance} = S^2 = \frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n - 1}$$

$$S^2 = \frac{0.003313}{19} = 1.7438 \times 10^{-4}$$

To find square root:

$$\log_{10} S = \frac{\log_{10} 1.7438 \times 10^{-4}}{2} = \frac{\bar{4}.24150}{2} = \bar{2}.12075$$

$S = \text{antilog } \bar{2}.12075 = 0.013205\%$ of vol. increase.

Table A1. Precision Assessment Data For The Gilson Respirometer Model GR 20

i	Measured Vol. (µl)	Observed Vol. Increase (µl)	% Vol. Increase x_i	Deviation $\lambda i = x_i - \bar{x}$	λ_i^2
1	22,900	26	0.1135	+ 0.0151	0.000228
2	21,200	23	0.1085	+ 0.0101	0.000102
3	23,400	26	0.1111	+ 0.0127	0.000161
4	20,200	17	0.0842	- 0.0142	0.000202
5	24,400	28	0.1148	+ 0.0164	0.000269
6	23,900	27	0.1130	+ 0.0146	0.000213
7	23,900	25	0.1046	+ 0.0062	0.000038
8	22,400	23	0.1027	+ 0.0043	0.000018
9	20,500	18	0.0878	- 0.0106	0.000112
10	24,100	27	0.1120	+ 0.0136	0.000185
11	21,000	19	0.0905	- 0.0079	0.000062
12	21,600	20	0.0926	- 0.0058	0.000034
13	24,100	27	0.1120	+ 0.0136	0.000185
14	24,400	25	0.1025	+ 0.0041	0.000017
15	20,900	18	0.0861	- 0.01[illegible]3	0.000151
16	20,900	15	0.0718	- 0.0266	0.000708
17	23,700	23	0.0970	- 0.0014	0.000002
18	21,000	16	0.0762	- 0.0222	0.000493
19	22,000	22	0.1000	+ 0.0016	0.000003
20	23,000	20	0.0870	- 0.0114	0.000130
	Σ		1.9679	- 0.0001	0.003313

Since $n = 20$, $\bar{x} = 0.0984\%$

Relative standard deviation = $\frac{0.013205\%}{0.0984\%} \times 100 = > 13\%$.

This analysis was based on the approach of Martin and Jordan (1971).

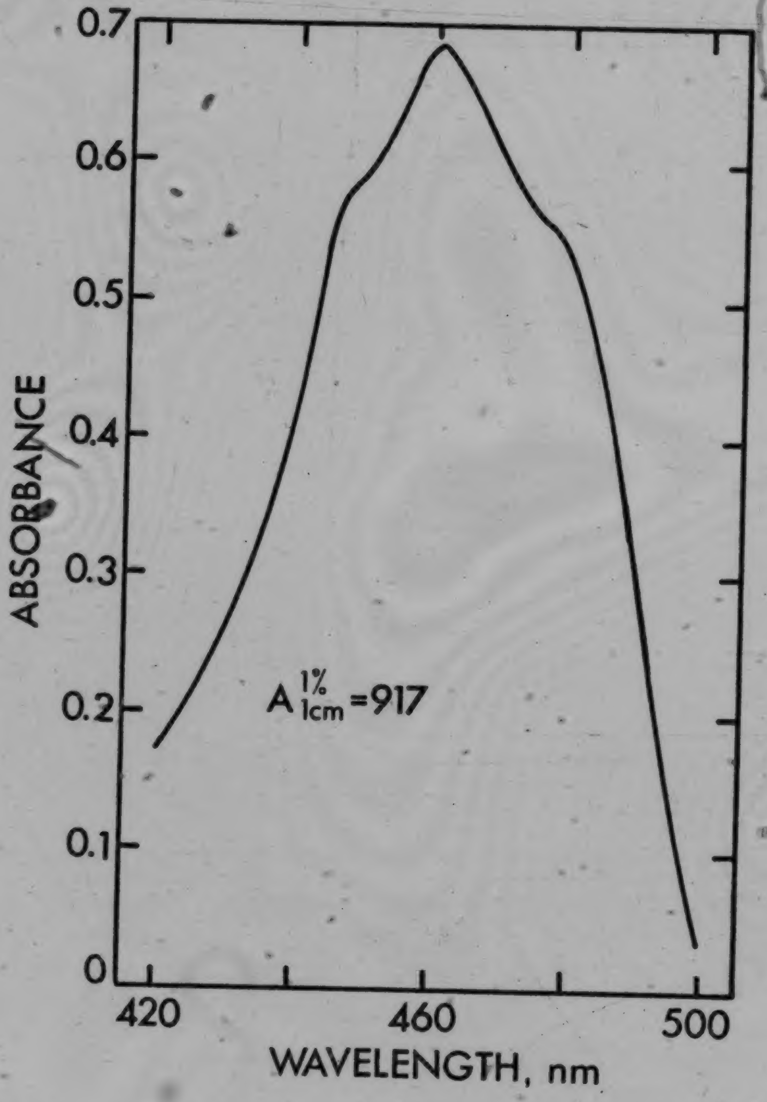

Figure A3. Facsimile of absorption spectrum of reaction mixture c, 3 parts + water, 1 part, totalling 3 ml (1.4×10^{-5} M β-carotene in aqueous buffered carotene - linoleate solution, pH 7.0, according to Ben Aziz *et al*., 1971)

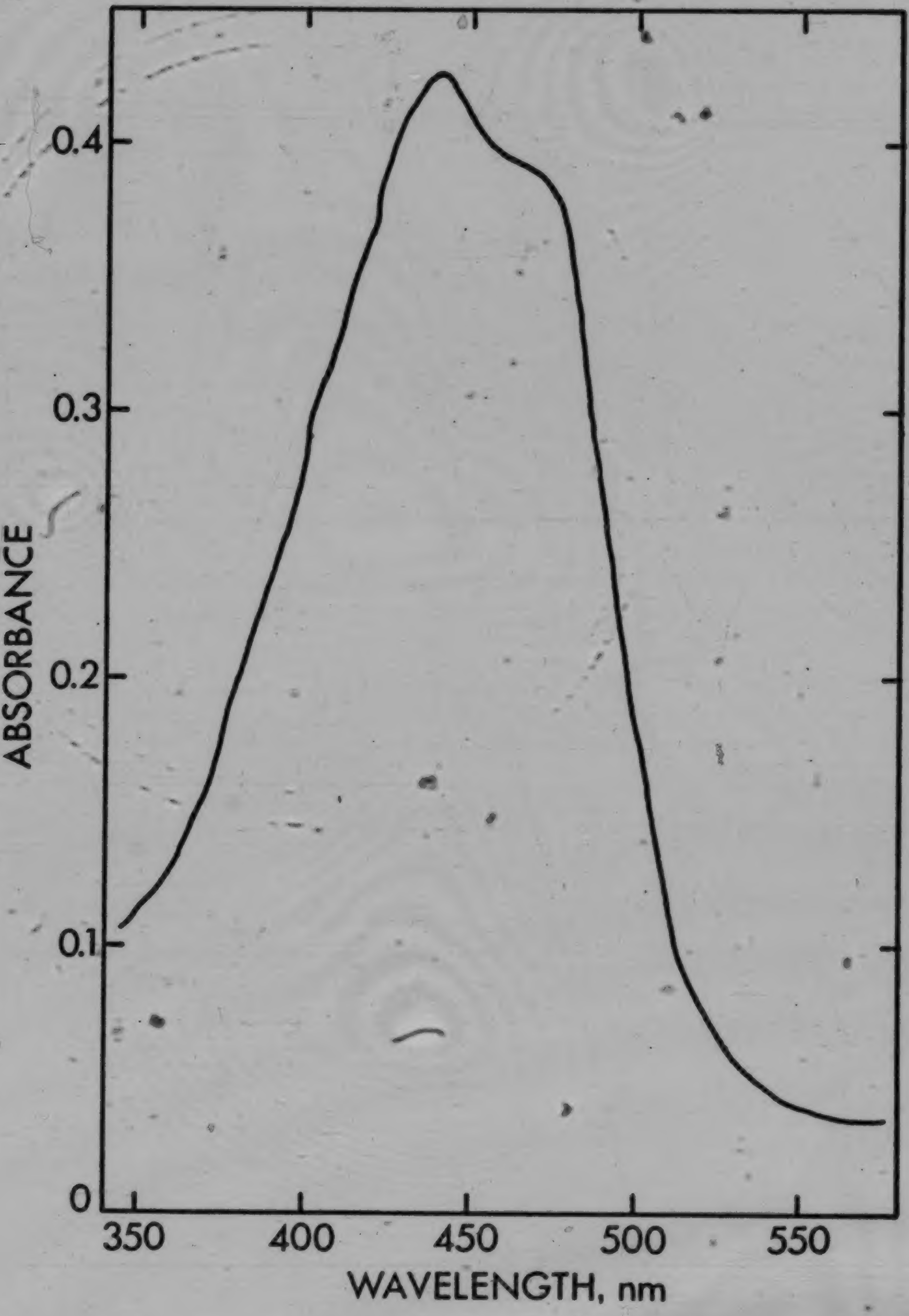

Figure A4. Facsimile of absorption spectrum of aqueous crocin.